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Role of small noncoding RNAs in mammalian adult neurogenesis

Supervisor: Dr. Davide De Pietri Tonelli

(NBT-IIT, Genova, Italy)

Caterina Gasperini

To all the "M" of my life

Preface

Neurogenesis is the process of new neuron (and glia) generation from Neural Stem Cells (NSCs). NSCs self-renew and generate committed offspring in a tightly regulated fashion. The balance between NSC proliferation and differentiation guarantees brain formation, lifelong neurogenesis and prevents tumor formation.

Regulation of neurogenesis is crucial but remains unclear. Understanding this regulation has implications for comprehending brain (mal)formation, maintenance of the capability to generate new neurons throughout life preventing **age-related disorders** and brain cognition.

Adult hippocampal neurogenesis attracts considerable attention from neuroscientists and the general public because of its suggestive appeal and presumed relevance for cognition in health and disease. It comprises a complex cascade of events, starting with the activation of quiescent resident NSC, followed by asymmetric cell division, rendering a new stem cell and a daughter neural progenitor. Neural progenitors then amplify through symmetric divisions and undergo cell fate decisions, whereas some cells are depleted through apoptosis. The surviving neural progenitors can then differentiate into immature neurons or astrocytes, which over time, will mature and integrate into the pre-existing neuronal network. Each of these steps in this cascade requires complex and rapid changes in the molecular machinery, which usually comprise multiple levels of molecular control.

The field of neurogenesis has been for long dominated by genetics, but protein-coding genes account for only 2 % of the human genome. In contrast, 98 % of the human genome encodes noncoding RNAs with gene-regulatory functions and nearly half of genome is comprised of Transposable Elements (TEs), which are highly active during neurogenesis. Moreover, **noncoding RNAs** are particularly relevant for the regulation of neurogenesis both in developing and in the adult brain. It follows that a better understanding of noncoding RNAs is essential to complete the puzzling mechanism of neurogenesis.

The aim of my PhD project is to better understand and characterize the role of two classes of small noncoding RNAs, namely microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) in the regulation of adult neurogenesis.

The work reported in my thesis provides **fundamental insight on the role of small noncoding RNAs in adult neurogenesis**: **First**, the discovery of a

crucial role of the Piwi-pathway in the maintenance of postnatal neurogenesis, opens the possibility to targets this pathway for therapy in the context of ageing and age-related brain dysfunctions, such as neurodegeneration. **Second**, the finding that inhibition of miR-135, a miRNA that is key mediator of physical activity, in 2 years old (equivalent to 70 years old human) sedentary mice is sufficient to rescue neurogenesis to young levels offers intriguing perspectives towards therapeutic uses of miRN-135 inhibitors to delay or prevent brain aging and related brain pathologies.

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Acronyms

Ago Argonaute
aNPC adult Neural Precursor Cell
aNSC adult Neural Stem Cell
Ascl1 Achaete-Scute homolog 1
BMP4 Bone Morphogenetic Protein
BrdU BromodeoxyUridine
CFP/CFPnuc Cyan Fluorescent Protein /nuclear
CNS Central Nervous System
DAB 3′-DiAminoBenzidine
DG Dentate Gyrus
DIV Days In Vitro
GO Gene Ontology
HBSS Hank's Balanced Salt Solution
KA Kainic Acid
KEGG Kyoto Encyclopedia of genes and genomes
miRNA microRNA
MOI Multiplicity Of Infection
ncRNA Non-Coding RNA
NGS Normal Goat Serum
NSC Neural Stem Cell
PFA ParaFormAldehyde
piRNA Piwi-interacting RNA
RPL Ribosomal Protein
rRNA Ribosomal RNA
SDS Sodium Dodecyl Sulfate
SEM Standard Error of the Mean
SGZ SubGranular Zone
SINE Short-INterspersed Element
siRNA small-interfering RNA
snoRNA Small Nucleolar RNA
STED Stimulated Emission Depletion
SVZ SubVentricular Zone
TE Transposable Element
TLDA TaqMan Low Density Array
tRNA Transfer RNA
U Uridine
UTR UnTranslated Region

Outline of this thesis

In the **introduction** chapter I will provide an overview of the three main topics of my project. I will first introduce NSCs, adult neurogenesis and regulatory mechanisms. Next, because small noncoding RNAs play an important role in this regulation, I will introduce miRNAs and piRNAs.

The **rationale and aims of the thesis** (chapter 2) outlines the purpose of research projects I have followed during my PhD and their potential impact in the field of adult neurogenesis.

In **chapter 3 and chapter 4** report the results I have obtained during the last three years, which have been published in [1] and in Gasperini et al., 2019 (Manuscript submitted) and I will discuss the main results obtained.

Results will be divided in two chapters, one dedicated to the piRNA project (chapter 3) and the second on the miR-135 project (chapter 4). In chapter 3, I will provide results of the first functional investigation of the Piwi pathway and piRNAs in somatic stem cells of the mammalian brain. In particular, our results indicate that Piwi proteins and piRNAs are enriched in aNPCs and have an essential role in in the regulation of adult neurogenesis. Specifically, the Piwi pathway sustains hippocampal neurogenesis and modulates global translational machinery. I will discuss how the piRNA-pathway in brain might be an "epigenetic immunity" required to maintain lifelong neurogenesis. In chapter 4, I will define the role of miR-135 in running-induced adult neurogenesis. By investigating miRNAs and their downstream pathways, we uncover that downregulation of miR-135a-5p mediates exercise-induced proliferation of aNPCs in adult neurogenesis in mouse hippocampus.

In the concluding remarks (chapter 5), I will place the main results of the two projects in the broader context of somatic stem cells, lifelong neurogenesis and ageing. In particular, I will focus on the possible applications of piRNAs and miR-135 to promote maintenance of the homeostasis of neurogenesis and in aging.

Part I

State of art

1 Introduction

1.1 Adult neurogenesis

For most of the 20th century, adult brain has been considered limited in its regenerative capacity, believing that neurogenesis desisted after birth. The discovery of adult neurogenesis was made in 1962, when Joseph Altman demonstrated that mitotically active progenitors exist in adult rat brain and give birth to new neurons [2]. Starting from this evidence, several studies have then showed that adult neurogenesis resides also in primates, including humans [3–5]. Neurogenesis is the generation of new neuron (and glia) from Neural Stem Cell (NSC), that can self-renew and generate committed offspring in a tightly regulated fashion. The balance between proliferation and differentiation guarantees brain formation, life-long neurogenesis and prevents tumor formation.

Adult neurogenesis consists in several distinct stages, each of which is regulated by gene expression and environmental factors granting tight temporal and spatial regulation of the process in a specific microenvironment [6]. Adult Neural Stem Cells (aNSCs) resident in the two main neurogenic niches of the adult mammalian brain: the SubVentricular Zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal Dentate Gyrus (DG), which is the only region capable of neurogenesis under basal conditions in mammals, including in humans (Figure 1.1).

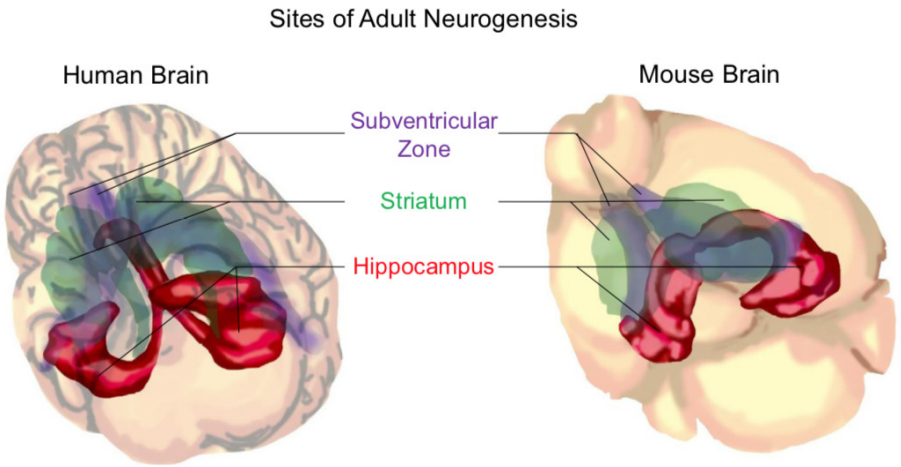


Figure 1.1: Adult neurogenesis regions. (**Left**) Human adult neurogenesis occurs under basal conditions in the hippocampus (red) and the striatum (green). (**Right**) Murine adult neurogenesis occurs in the hippocampus (red) and the subventricular zone (purple). The hippocampus serves as the only brain region where adult neurogenesis is conserved across mammalian species [7].

aNSCs in the DG have the capacity to self-renew and differentiate, giving rise to both neurons and glia [8, 9]. aNSCs are radial-glia like cells mainly quiescent, but they can re-enter the cell cycle and become proliferative. aNSCs express nestin, GFAP and Sox2 and possess a defining radial branch extending through the granule cell layer. Once activated, aNSCs can self-renew or give rise to astroglia (s100β, GFAP and Sox2 positive cells) or early amplifying neural progenitors (aNPCs). These cells undergo multiple rounds of symmetric division, to expand the neurogenic pool and to mature in neurons (expressing DCX and Prox1, markers of committed immature and NeuN later on during differentiation) (Figure 1.2).

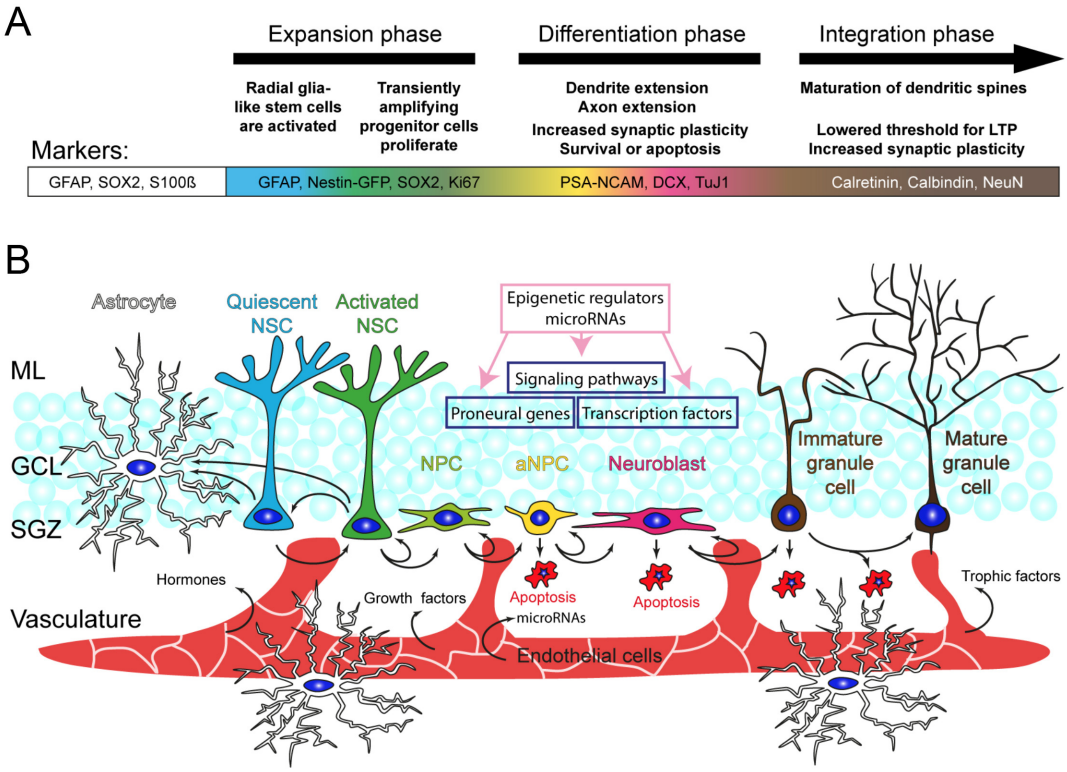


Figure 1.2: Hippocampal neurogenesis niche and aNSC processes. (**A**) Different stages of adult hippocampal neurogenesis with markers specific for each cell type. (**B**) Overview of the neurogenic niche and the transition of a NSC into a mature neuron. Modified from [10].

Many aNPCs die shortly after birth by apoptosis, to avoid an excessive production of new or unfit neurons [11, 12]. The remaining aNPCs become neuroblasts and, after neuronal differentiation, migration, and maturation, they will integrate into the DG network. Adult neurogenesis in the hippocampus can generate only one type of neuron: granule cells that are the excitatory principal neurons of the dentate gyrus [13, 14].

The composition and functionality of the neurogenic niches has been most extensively studied in rodents [6, 15]. Similar regions containing neurogenic progenitor cells have been described in the SVZ and DG of the adult human brain [4, 16, 17]. The neurogenic capacity of aNSCs decreases throughout life, mainly due to a decline in proliferation and a loss of NSCs, probably through astrocytic conversion [18]. Neurogenesis occurring in the human SVZ declines during infancy [19], whereas several studies have pointed to a quite substantial generation of DG neurons in humans throughout life [20, 21].

Regulation of neurogenesis is crucial but remains unclear; understanding this regulation has implications for comprehending brain (mal)formation, lifelong neurogenesis and preventing age-related disorders.

1.1.1 Regulation of adult neurogenesis

There is a strong interest in understanding the mechanisms regulating adult neurogenesis: research is motivated by the fact that the hippocampus is involved in memory and learning, which led to the hypothesis that adult neurogenesis could play an important role in cognition [7, 22].

Hippocampal neurogenesis can be detrimental or beneficial to disease outcome (Figure 1.3). Indeed, altered hippocampal neurogenesis has been linked to a number of pathological conditions, such as ischemia- or epilepsy-induced insults, mood disorders and neurodegenerative diseases [15]. Aging is also influencing adult neurogenesis: age-related neurogenesis decline coincides with an increasing incidence of neurodegeneration and a decreased regenerative capacity after injuries [23].

On the other side, adult neurogenesis can act as a beneficial contributor for treatment and symptom amelioration in depression [24]. Moreover, it is known that neurogenesis contributes to more efficient repair and regeneration during stroke and traumatic brain injury [25, 26].

A series of studies revealed that adult hippocampal neurogenesis in rodents can be modulated by experiential and environmental conditions as well as by aging [27]. These studies strongly suggest that adult hippocampal neurogenesis is a key regulator for disease progression and can be used as a target for therapeutic drugs (reviewed in [7]).

The mechanisms of fate determination in aNSC lineage is a highly debated topic [28, 29] of fundamental importance. Indeed, understanding the molecular mechanisms underlying lineage determination might provide new avenues to prevent age-dependent loss of neurogenesis [18, 30, 31], or the pathological generation of undesirable cells such as activated glia upon trauma and epilepsy [32–34].

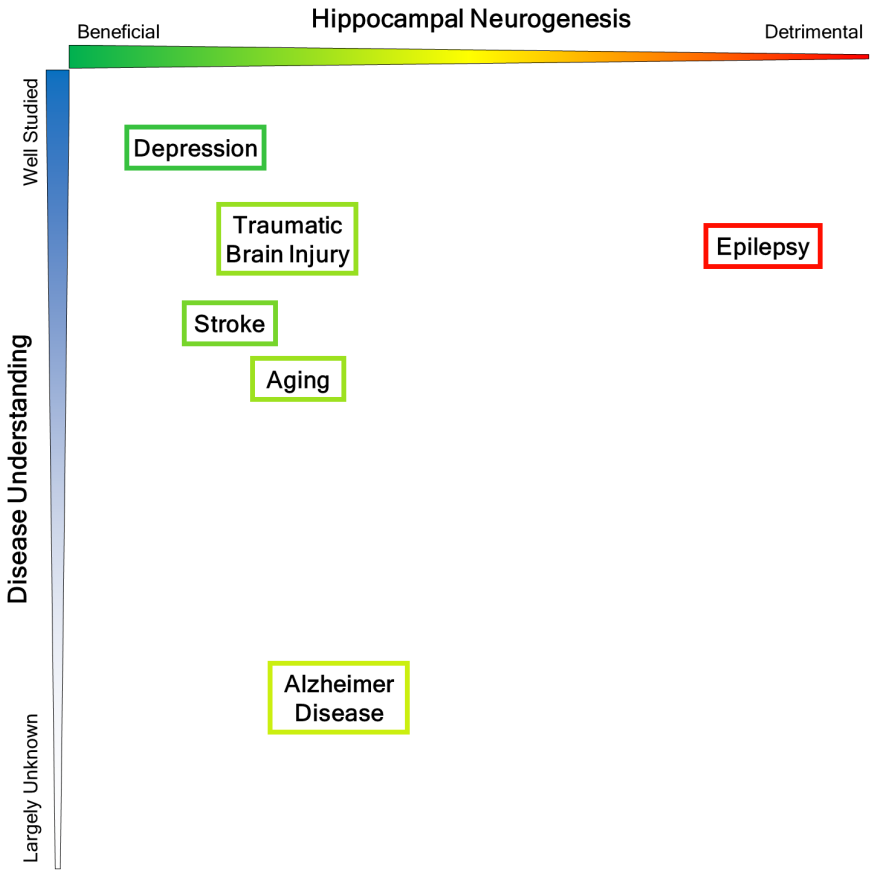


Figure 1.3: Roles of adult neurogenesis in disease. Hippocampal neurogenesis can be beneficial or detrimental to disease outcome, with varying degrees of data to support these interpretations [7].

1.1.2 Epigenetic control of adult neurogenesis and noncoding RNAs

Adult neurogenesis involves multiple steps that have to be tightly regulated, *i.e.*, aNSC activation, proliferation, differentiation into neural progeny as well as survival, migration, and functional maturation of the adult-born neurons. Regulation of aNSC fate determination is known to be possible at the transcriptional level [35], but accumulating evidence indicates that additional control layers, such as epigenetics and noncoding RNAs, are involved in this mechanism [36–40].

Regulation of aNSC fate determination is known to be possible at the transcriptional level, but accumulating evidence indicates that additional control layers, such as epigenetics and noncoding RNAs, are involved in this mechanism. Among the most commonly used definitions, epigenetic is the study of changes in gene function that are mitotically and/or meiotically heritable and

that do not involve a change in DNA sequence [41]. In this sense, genotypically identical cells can behave phenotypically different thanks to epigenetic alterations in chromatin organization and/or biochemical changes. The dynamic nature of epigenetic mechanisms provides a crucial layer of gene regulation, controlling adult neurogenesis in response to environmental signals. As shown in Figure 1.4, there are four major categories of epigenetic mechanisms, which function as key regulators of gene expression also in adult neurogenesis: chromatin remodeling, histone modification, DNA methylation and noncoding RNAs (ncRNAs)Figure 1.4.

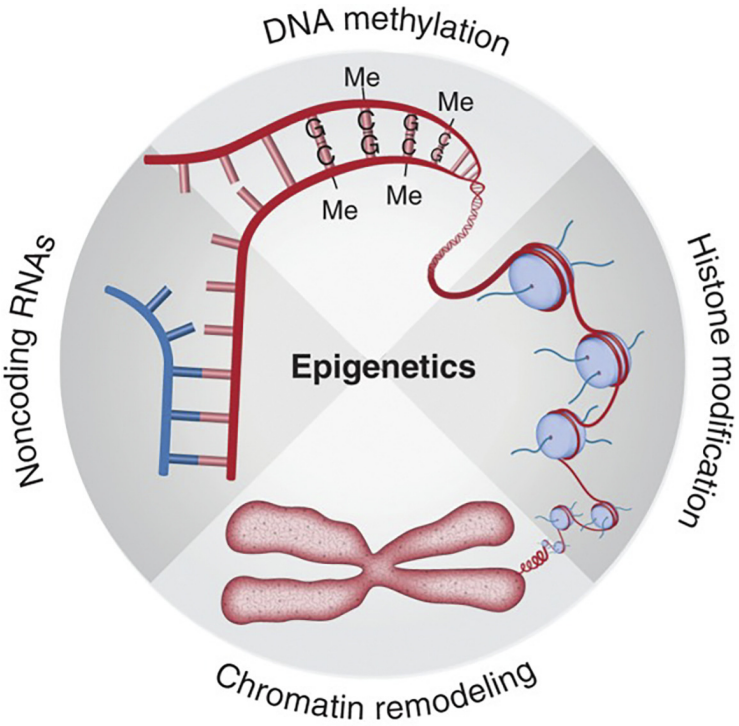


Figure 1.4: Epigenetic mechanisms. Four major categories of epigenetic mechanisms: histone modification, chromatin remodeling, DNA methylation, and noncoding RNAs (ncRNAs) [42].

Even if the main focus of my thesis will be this last category, a brief overview of the other mechanisms is worth, given their relevance in the regulation of neurogenesis. Several studies have shown how chromatin remodeling due to the many types of histone modifications occurring on different histone residues, contributes to the regulation of neuronal differentiation, survival, and maturation. For example, inhibition of histone deacetylase is able to induce neuronal differentiation of adult hippocampal neural progenitors [43]. Despite this study, the function of individual histone deacetylases in adult neurogenesis is largely unresolved and more studies are required. However, it is tempting to speculate that pharmacological inhibition of their activity might become an effective clinical

strategy for treating disorders related to adult neurogenesis, such as cognitive decline, and promote neural repair [42]. Another example is the knockdown of lysine-specific demethylase 1 (LSD1) in cultured adult NPCs and in adult mouse DG, which resulted in a dramatically reduction of neural stem cell proliferation [44].

Epigenetic modifications implicated in adult neurogenesis comprise also DNA methylation. This modification is a major epigenetic mechanism for the establishment of parental-specific imprints during gametogenesis and gene silencing of the inactivated X chromosome and retro- transposons [45], but it has been shown to be crucial also during neurogenesis. Indeed, during the neural induction of embryonic stem cells (ESCs) to NPCs, many pluripotency genes are methylated and silenced [46]. Moreover, DNA methyl-transferases (DNMT) 3a and 3b appeared to be essential for specification of neurons and glia, during the early phase of neurogenesis [47], and during later stages of neuronal maturation and function [48, 49]. Another DNMT, the 1, is involved in JAK-STAT signaling to control the timing of when precursor cells switch from neurogenesis to gliogenesis during development. Through a chromatin remodeling process, demethylation of genes in the JAK-STAT pathway leads to an enhanced activation of STATs, which in turn triggers astrocyte differentiation [50].

Lastly, among the epigenetic mechanisms, ncRNAs play an essential role in adult neurogenesis. Recently, long noncoding RNAs (lncRNAs) have been linked to regulation of stem cells and neural plasticity, although their mechanisms of action are diverse and largely unknown [51, 52]. lncRNAs size is between 200 to 10 000 bases and may undergo splicing and polyadenylation, similar to protein coding mRNAs [53]. In the human genome there are more than 30 000 lncRNAs and it seems that at least half of these are expressed in the Central Nervous System (CNS). Moreover, some of them show dynamic expression patterns during cellular differentiation and brain development [54].

On the other hand, small noncoding RNAs have been extensively studied in neurogenesis, given their small size and sequence complementarity, which allow extreme versatility to target mRNAs for regulation of gene expression or chromatin structural modification of targeted genes [42]. Indeed, several researches have revealed small RNA pathways as key regulators of diverse types of stem cells, including neural stem cells [55].

Small noncoding RNAs can regulate gene expression by guiding Ago protein-containing complexes in a nucleotide sequence-specific way to different sites for molecular actions [56] (Figure 1.5). The Ago protein family is divided into the Ago and Piwi subfamilies, based on phylogenetic analysis. Ago subfamily proteins bind miRNAs and small-interfering RNAs (siRNAs), which are both

made from double-stranded precursors. Ago subfamily proteins are ubiquitously expressed in animal tissues and miRNAs and siRNAs bind to them to repress gene expression, by promoting heterochromatin formation, mRNA turnover and translational repression [57].

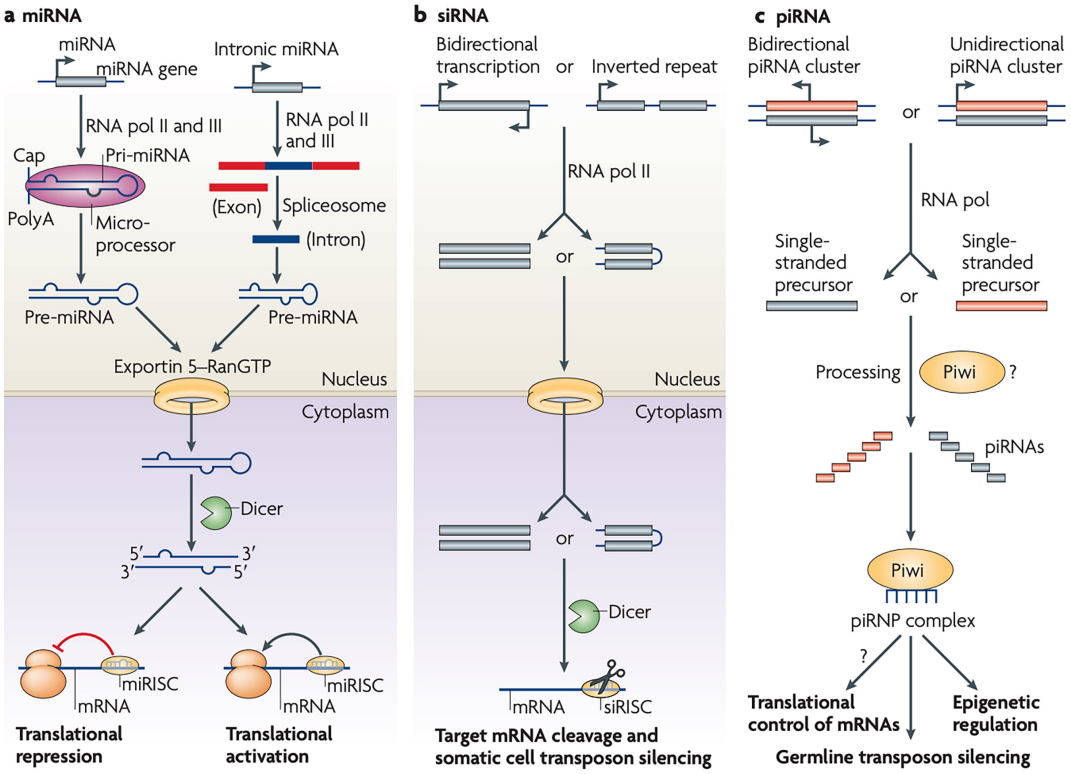


Figure 1.5: Small noncoding RNAs interacting with Ago proteins. Biogenesis and regulatory features of the miRNA (**a**), siRNA (**b**) and piRNA (**c**) pathways [55].

Among ncRNAs, miRNAs are the best studied both in terms of their expression profiles and functions: a number of miRNAs are known to play important roles in stem cells and development. For example, miR-9 and miR-124 are enriched in the brain compared with other tissues. In fact, they are among the most abundant miRNAs in the CNS.

In contrast, Piwi protein expression is mostly restricted to germ cells and stem cells [58]. Piwi proteins bind to piRNAs which are processed in a Dicer-independent manner from long single-stranded precursors [59–61]. piRNAs regulate gene expression at the epigenetic, post-transcriptional and translational levels. Studies on animals from diverse *taxa* also demonstrate that Piwi proteins have a conserved function in stem cells. Therefore, understanding how Piwi proteins and piRNAs regulate gene expression could be the key to understand the regulation of stem cells identity, maintenance and differentiation, not

only in the germline but also in the brain neurogenesis [62].

1.2 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a class of small RNAs, with an average 22 nucleotides in length, that bind to specific mRNA targets, directing their degradation and/or repressing their translation [63, 64].

About half of all identified miRNAs are intragenic and processed from introns (the majority of them) and from exons (relatively few) of protein coding genes. The other half comprises intergenic miRNAs, transcribed independently of a host gene and regulated by their own promoters [65, 66].

In most cases, miRNAs suppress target mRNAs expression interacting with their 3′UnTranslated Region (UTR). However, miRNA binding sites have been detected in other mRNA regions, like the 5′UTR, the coding sequence and within promoter regions [67]. miRNAs exert their functions in a highly combinatorial way: one single miRNAs can regulate expression of multiple target genes and biological processes [68], but also different miRNAs can target one gene simultaneously, repressing its expression in a more efficient way [69].

1.2.1 Biogenesis of miRNA

miRNA biogenesis starts with the processing of RNA polymerase II/III transcripts post- or co-transcriptionally [70]. miRNAs are transcribed from DNA sequences into primary miRNAs (pri-miRNAs); some pri-miRNAs are polycistronic and encode for diverse mature miRNAs [71]. In the nucleus, pri-miRNAs are then processed into precursor miRNAs (pre-miRNAs) by the Microprocessor protein complex, which encloses Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) proteins. Pre-miRNAs are translocated in the cytoplasm and further processed into mature miRNAs by Dicer, an RNase III-like enzyme; studies on the genetic ablation for Dicer have been used to determine the importance of the miRNA system. Mature miRNAs are then loaded onto Ago proteins to form the RNA-induced silencing complex (RISC) through which they perform translational inhibition or mRNA degradation of specific targets [72].

miRNAs can be produced also by non-canonical pathways, Drosha- or Dicer-dependent. A non-canonical pathway was first described to take when miRNAs originate from debranched introns that mimic the structural features of pre-miRNAs to enter the miRNA-processing pathway omitting Drosha cleavage. These non-canonical miRNAs have been termed mirtrons (pre-miRNAs/introns) [73]. Drosha-mediated processing is also bypassed in the cases of small RNAs derived from endogenous short hairpin RNAs, which are generated directly

through transcription and are further processed into miRNAs by Dicer [74]. Although most alternative miRNA pathways depend on Dicer, biogenesis of miR-451 does not require Dicer and involves the catalytic activity of AGO2. This miRNA is processed by Drosha and the pre-miRNA is directly loaded into Ago and is cleaved by the Ago catalytic centre [75, 76] (Figure 1.6).

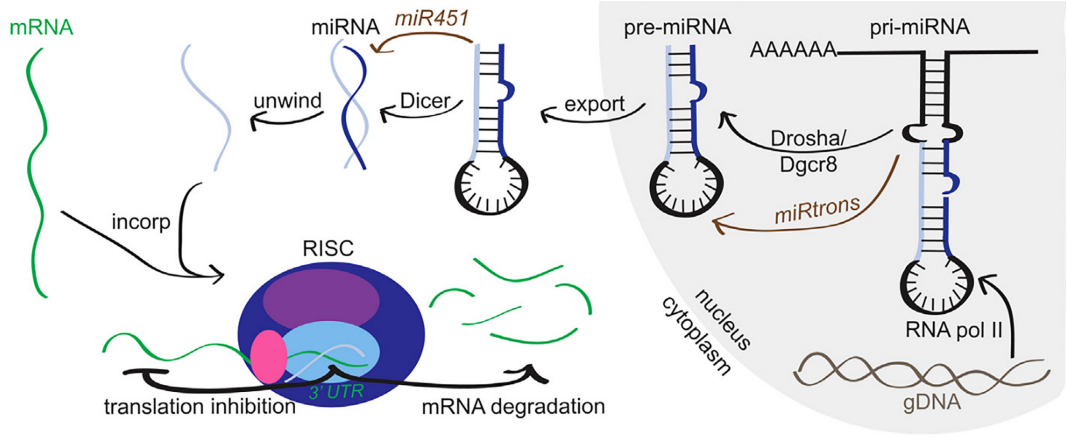


Figure 1.6: miRNA biogenesis and function. In the nucleus, pri-miRNAs are transcribed by RNA polymerase II (RNA pol II). The canonical miRNA pathway is thereafter followed by Drosha/Dgcr8 cleavage into pre-miRNA. miRtrons are known to skip this step and are instead processed through intron splicing and lariat debranching. After exportation of the pre-miRNA into the cytoplasm, the canonical pathway continues with cleavage by the RNA III nuclease Dicer into mature miRNAs. For the miRNA miR-451, this cleavage is performed by Ago2 rather than Dicer. After unwinding of the mature miRNA, one strand is incorporated into the RNA- induced silencing complex (RISC) and recruits its target mRNAs. This binding is dependent on sequence complementarity in the 3′-untranslated region (3′UTR) of the mRNA. Successful RISC processing results in translation inhibition or degradation of the target mRNA [77].

The existence of alternative pathways reflects the evolutionary flexibility of miRNA biogenesis. However, the majority of functional miRNAs are produced by the canonical pathway, and only about 1 % of conserved miRNAs (*i.e.* miR-320 and miR-451) are produced independently of Dicer or Drosha in vertebrates [70]. However, canonical miRNAs in NSCs of developing mouse are only 58 % of total mature miRNAs, suggesting that miRNA biogenesis is more complex in the development of the nervous system, compared to other tissues [78].

Although this is not the focus of my thesis, it has to be mentioned that DGCR8 and Drosha can have alternative functions which include, but are not limited to non-canonical alternative miRNA biogenesis pathways. Accumulating evidence indicate that the Microprocessor mediated cleavage regulate mRNA levels and also modulate alternative-splicing events [79]. Furthermore, DGCR8 promotes cortical NPC self-renewal and repress their differentiation *in vivo* [78, 80].

1.2.2 miRNAs in adult neurogenesis

Approximately 70 % of known miRNAs are expressed in the mammalian brain [81], and the level of many miRNAs changes dramatically during brain development [82–84]. Indeed, based on observations obtained with cell culture models in vitro, miRNAs have been implicated in the control of neural progenitor proliferation, neurogenic and gliogenic differentiation, maturation and functional integration of postmitotic neurons [72, 85–90].

Furthermore, miRNAs play important roles in the regulation of cell fate decisions in the adult SVZ and SubGranular Zone (SGZ) [91, 92] and have been linked to several diseases associated with these areas, *e.g.*, epilepsy [93], stroke [94], and neurodegenerative disorders [95].

The functional synergism of few miRNAs achieves gene regulation essential for proliferation, cell fate determination, and survival in embryonic [89] and adult NSCs [72,96,97]. Cooperation between co-expressed miRNAs might compensate the fine-tuned mRNA regulation mediated by a single miRNA, thus exerting a broader impact on gene expression compared to a single miRNA. The positive interaction of two or more individual miRNAs, or one individual miRNA, acting on multiple seed regions on the same 3′UTR, is described as "miRNA cooperativity" [98]. In this context, our lab have shown how the cooperativty of 2 miRNAs on the gene Foxp2 regulate the plasticity of the embryonic mouse neocortex [99].

In recent years multiple miRNAs have been identified to play a crucial role in the transition between proliferative and differentiating state [10]. Moreover, several studies suggest that miRNAs play a crucial role in ensuring proper numbers of aNPCs by either directly silencing target genes, or forming a regulatory loop with targets [100, 101]. Accumulating evidence indicates that most of the miRNAs can be divided in two groups, based on their roles: they promote either proliferation, such as the miR-17-92 cluster, or differentiation such as miR-9 and miR-124 (Figure 1.7).

However, the diversity and complexity of individual miRNAs in cell fate determination appear to rely on different species, specific regions in the nervous system, distinct cell context, and mostly the availability and direct physical interaction of their target genes. (reviewed in [102]).

In many cases, miRNAs can also act in concert with transcription factors and chromatin modifiers to control gene expression in NSCs, thereby affecting NSC number and their ability to generate differentiated progeny.

By doing so, miRNAs provide an additional layer to control gene expression

programs and may help to ensure the robustness of such programs by buffering perturbations and reducing noise [91, 92, 103, 104]. However, miRNAs may even exert an instructive effect on cell fate as impressively demonstrated by the finding that miR-9/9* and miR-124 can induce neuronal conversion of fibroblasts [105].

Furthermore, global miRNA loss by Dicer depletion seems to evoke stronger effects in differentiating cells than in self-renewing NSCs (derived from either embryonic and adult origin), suggesting that cell fate transitions show a particular dependency on miRNA-based regulation [89, 90, 97].

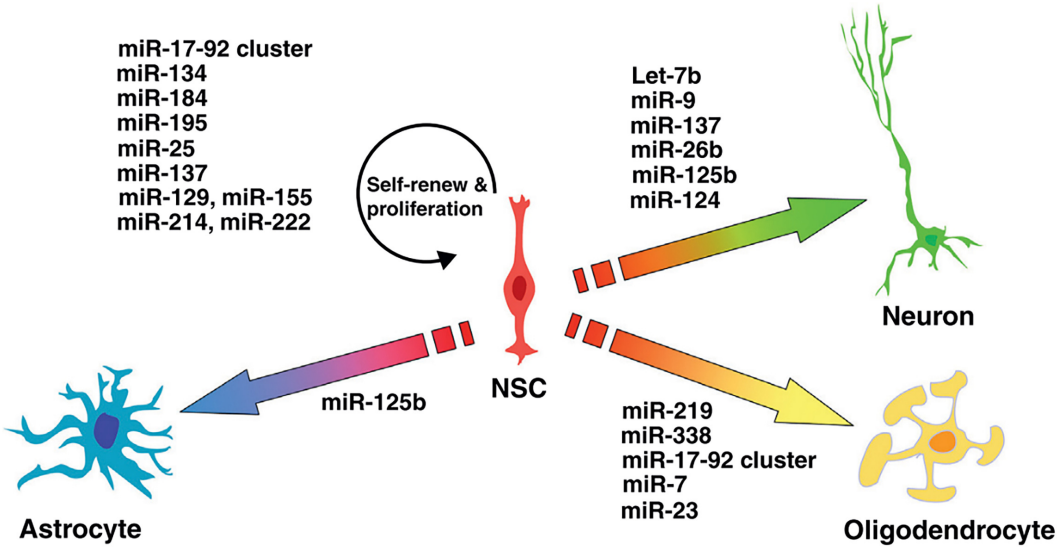


Figure 1.7: Scheme of the roles of miRNAs in cell fate determination. List of miRNAs regulating NSC self-renewal and proliferation, neuronal differentiation, astrogliogensis, and oligodendrocyte differentiation [102]

1.3 Piwi proteins and piRNA

P-element-induced wimpy testis (Piwi) proteins are predominantly nuclear proteins that represent a large subfamily of Ago proteins and are expressed widely from ciliates to mammals. Their expression is mostly restricted to the germline: different Piwi proteins exist and function in different stages of the germline cycle. The prototype of Piwi proteins is encoded by the *Drosophila* piwi (P-element-induced wimpy testes) gene, originally identified as an essential gene for germline development [58, 106]. In *Drosophila* there are three Piwi genes: ago3, aubergine (aub), and piwi. Piwi and aub are required for both male and female fertility, whereas ago3 is essential for female fertility [107, 108].

The mouse genome expresses Miwi (Piwil1), Mili (Piwil2) and Miwi2 (Piwil4),

but it lacks one of the four Piwi paralogs that is found in most other mammals, including primates and humans, Piwil3 [109]. In mice, Piwi proteins are expressed at different stages during spermatogenesis. Strikingly, none of the murine Piwi proteins affect female fertility [110–112]. However, the expression of Miwi and Mili has been detected in mouse oocytes, but they present only relatively minor amounts of piRNAs [113, 114].

The human genome encodes four Piwi proteins, HIWI (Piwil1), HILI (Piwil2), HIWI3 (Piwil3) and HIWI2 (Piwil4). Given the conserved functions of Piwi proteins in stem cell maintenance and germline development, most of the studies on human Piwi proteins are carried out in germ cell-derived tumors. Thereafter, it has been shown that Piwi proteins are expressed in a large number of human cancers, of both germline and somatic origin, such as seminomas, prostate, hepatocellular, multiple myeloma, gastrointestinal, ovarian, breast and endometrial cancer [115–117]. In some of these cancers, the expression of Piwi proteins correlates with a significant worse prognosis. Even if the number of studies on human Piwi proteins in somatic tissues is increasing, especially in pathological conditions, their presence and role in brain remains still obscure.

Among different organisms, the crucial role of Piwi proteins can be found from the earliest stage of germline development (germline fate specification) to late stages of gametogenesis such as spermiogenesis, egg activation, and fertilization [58]. Nonetheless, Piwi proteins were first characterized in somatic cells of the *Drosophila* ovary and in early somatic cells of the testis [118]. Somatic function of Piwi proteins is evident during early embryogenesis: embryos laid by piwi mutant mothers, are somatic lethal [118, 119]. Moreover, Piwi was identified as essential for Germline Stem Cell (GSC) maintenance in *Drosophila* [108] by mediating a highly conserved somatic signaling mechanism [106].

Piwi-interacting RNAs (piRNAs) are a novel class of noncoding RNAs which biogenesis and function depend on Piwi proteins. piRNAs are single stranded small non-coding RNAs consisting of 24-31 nucleotides, with a phosphorylated $5'$ end and a $2'$ O-methyl ($2'$ O-me) modification at their $3'$ ends [61,120]. piRNAs associate with Piwi proteins to form effector complexes, piRNA-induced silencing complexes (piRISC), which maintain germline genome integrity repressing mainly transposable elements (TEs) but also genes, with a transcriptional or posttranscriptional mechanisms [121].

Transposon regulation by piRNAs is conceptually similar to that in immune systems, which can achieve “self” and “nonself” recognition. Indeed, piRNAs use a complex mechanism to effectively select the nonself genes for regulation, as with our immune systems [122]. Moreover, analyses of various eukaryotes identified piRNAs targeting protein-coding genes and piRNAs that are passed

through generations to transmit a memory of past transposon activity [123,124].

Even if transposons are the major targets of Piwi–piRNA pathway, how regulation of transposons is connected to defects in gametogenesis is still unknown. Activation of transposons in Piwi protein mutants could lead to the generation of double-stranded DNA breaks within abortive or successful transposition, activating DNA damage checkpoint which results in a sterile phenotype [125]. Thus, tissue-specific and developmental timing–specific expression of Piwi proteins and piRNAs may play major roles in maintaining the integrity of the genome and fertility of the organism.

1.3.1 Biogenesis of piRNA

piRNAs are distinct from other noncoding RNAs such as miRNAs and siRNAs, for their biogenesis and function. miRNAs and siRNAs processing from their precursors requires cleavage by Dicer before loading onto Ago protein, instead piRNAs are processed through Dicer-independent mechanisms [59, 61, 126].

The great majority of piRNAs is transcribed from genomic clusters of 1 to 127 kb, often within intergenic sequences. Initial analysis of piRNA sequences revealed their extremely high complexity: there are hundreds of thousands, if not millions, of individual piRNA sequences. Indeed, although the genomic locations of clusters are conserved, there is very little conservation at the level of individual piRNA sequences [61, 126].

Extensive analyses of piRNAs associated with Piwi proteins in flies and mice had identified the genomic origins of piRNAs and led to the proposal of two biogenesis pathways, summarized in Figure 1.8: in the nucleus long piRNA precursors are transcribed from piRNA clusters or transposon loci and processed in the cytoplasm; here, piRNA precursors can enter in the primary pathway, where they are processed as mature piRNAs and loaded into the Piwi proteins (Miwi or Mili, according to their size). In the cytoplasm, piRNA precursors can also enter in the secondary process, where piRNAs derived from sense or antisense strand can be loaded into Mili or Miwi2 protein and perform the so-called "ping-pong cycle", where the pool of piRNAs is amplified according to the transposable elements (and possibly the target mRNAs) present in the cytoplasm [122, 127]. In the ping-pong cycle, Piwi proteins utilize their slicer activity to cleave antisense retrotransposons and generate new antisense piRNAs, which are immediately bound by another Piwi protein. In subsequent steps, the antisense piRNA is trimmed to the length of the mature piRNA, leading to a mature antisense secondary piRNA, which can, in turn, target sense retrotransposon [122, 128].

Primary piRNA-directed cleavage of transposon mRNAs creates the 5′ ends of

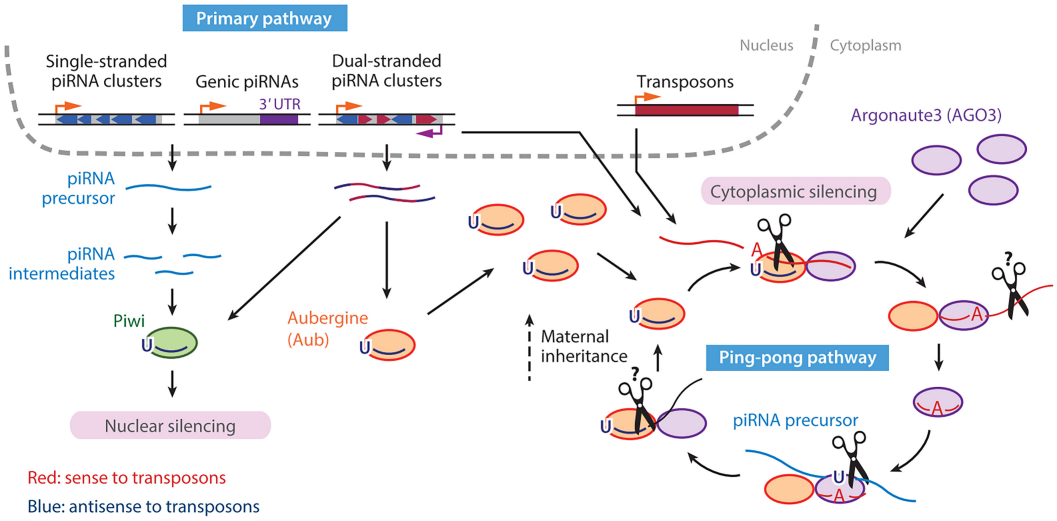


Figure 1.8: piRNA biogenesis. In the primary pathway, piRNAs are transcribed either from genomic regions called piRNA clusters, 3′UTR of protein-coding genes or transposons, processed, and loaded onto Piwi (Miwi) or Aub (Mili). Silencing takes place both in the cytoplasm and nucleus. Together with AGO3 (Miwi2), the Aub(Mili)–piRNA complex serves as a trigger to start the ping-pong amplification pathway. The ping-pong pathway silences the target transposon sequence and amplifies the piRNA sequence at the same time [121].

secondary piRNAs. This produces primary and secondary piRNA pairs that overlap by 10 nucleotides at their 5′ ends. The 5′ Uridine (U) bias of primary piRNAs thus leads to an enrichment of an A at position 10 of secondary piRNAs (Figure 1.8) [129]. Both mechanisms are important to form an active piRNA-induced silencing complex (piRISC) that can recognize and silence complementary RNA targets, providing an effective defense against transposons. In animals, endogenous siRNAs also silence TEs, but the piRNA pathway is at the forefront of defense against transposons in germ cells. Indeed, in mice the role of Piwi proteins and piRNAs seems to extend beyond post-transcriptional silencing: evidence indicates that Piwi and piRNAs can play a crucial part in epigenetic regulation. CpG DNA methylation, which is required for efficient transcriptional silencing of TEs such as LINE and LTR, is decreased in the male germline of Mili and Miwi2 mice mutants. Genetic evidence suggests that both mutants fail to establish *de novo* methylation of TE sequences during spermatogenesis, leading to the hypothesis that the piRISC can also guide the *de novo* methylation machinery to TE loci [130–132].

1.3.2 piRNAs in Central Nervous System

Since its discovery, somatic functions of the Piwi pathway have long been documented. Originally, studies carried out in *Drosophila* showed that the role of Piwi genes in germline depends on the somatic cells of the gonad [106]; later on

several works reported an essential activity of Piwi proteins in stem cells [124]. Furthermore, in the CNS, piRNAs expression were firstly detected in neurons of the sea slug *Aplysia* [133]. Lower eukaryotes, such as *Drosophila* and *Aplysia*, constitute an excellent arena for the study of Piwi pathway, allowing to make rapid progress on this topic. Therefore, several studies on rodents and primates reported the expression of Piwi proteins and piRNAs in mammalian brain, and their role in suppressing transposons [134–139].

It has been long considered that the majority of retrotransposition events occur in the germline, while the mobility of TEs in somatic cells is strictly suppressed. In the last few years, however, sufficient data have been accumulated suggesting that the TEs activity may be a common property of cells in somatic tissues, even in brain [140]. Indeed, L1 (Long Interspersed Nuclear Elements 1) retrotransposons have been studied widely in the CNS, showing that the neuronal genomes are not static and might be mosaic because of *de novo* L1 retrotransposition events [141]. Moreover, studies revealed that the L1 expression increase upon neuronal commitment and its retrotransposition affects the expression of neuronal genes during neurogenesis [142,143]. Retrotransposition events caused by misregulated mobile elements were also reported to occur in autism and other neurological disorders [144, 145].

However, an increasing knowledge about TEs is not leading to a complete comprehension of the Piwi pathway functions in brain. Main limitation of these studies is that Piwi proteins and piRNAs are low expressed in mammalian brain compared to gonadal tissue.

piRNAs expression was first reported in mammalian nervous system by Lee et al. in 2011. Using high throughput sequencing of small RNA libraries, they identified piRNAs in adult mouse hippocampus. These sequences were intergenic with unique genome location. They reported the presence of abundant piRNA complexes in the dendritic spines; knockdown of piRNAs resulted in reduced spine density in the axons. Miwi expression was proven in hippocampus by western blot, *in situ* hybridization and qRT-PCR. The expression of Mili and Miwi2 was not detected in their study [134].

Furthermore, the same year, Dharap et al. showed that stroke rapidly alters the cerebral piRNA profiles in rat model. They noticed that the expression levels of 105 piRNAs were significantly altered in the cerebral cortex of rats subjected to transient focal ischemia [136]. One year later, studying Rett syndrome, Carninci lab demonstrated that the absence of a functional MeCP2 in mouse cerebellum leads to an increase in the total piRNAs, and this over-expressed piRNAs function not only to silence retrotransposons, but also to fine tune the expression level of specific genes [145].

Latterly, Zhao et al. (2015) revealed the expression of Miwi in cortical tissues of developing mouse brain and its possible role in promoting neuronal polarization and radial migration, partly via modulating the expression of microtubule-associated proteins (MAPs). Considering the known function of Piwi proteins in suppressing transposons, an interesting future question is whether MAPs are the regulatory target of L1 retrotransposons, which are controlled by Piwi and piRNAs: thus, this hypothesis lead to investigate whether Miwi promotes the expression of MAPs indirectly by processing the mRNAs of retrotransposons and suppressing their activity [137].

More recently, Carninci and Ravasi group conducted an almost exclusively computational investigation for the identification of piRNAs in adult mouse brain. In this study they identified small RNA molecules that exhibit the hallmarks of piRNA such as length and uridine bias at the first position. They demonstrated that these piRNAs were similar to Mili-bound piRNAs with regards to their length (26-27 nt) and, moreover, they predicted novel candidate regulators and potential targets of piRNAs in such system [135].

In humans, recent studies reported dysregulation of piRNA expression in Parkinson's disease (PD), AD and neurodegenerative tauopathies. Schulze et al., showed that Short-INterspersed Element (SINE)- and LINE-derived piRNAs are highly downregulated in fibroblasts, induced pluripotent stem cells (iPSCs) and neuronal cells derived from patients with PD, together with an increased expression of TEs [146]. In AD patients, transcriptome-wide piRNA profiling of human brains revealed 103 piRNAs differentially expressed in the pathological cases compared to healthy controls [147]. Moreover, Mallick Lab in 2017, found that 1923 mRNAs were significantly down-regulated in subject with AD and were predicted targets of 125 up-regulated piRNAs [148]. More recently, Sun et al. showed that reduced levels of Piwi proteins and piRNAs in neurodegenerative tauopathies, drive TEs activation and promotes neuronal death [149].

Despite these evidences, functions and regulation of the Piwi pathway in the central nervous system are still unclear and controversial. Given the increased expression of TEs during neurogenesis [141, 150], it would be plausible to hypothesize that piRNAs might constitute a negative feedback loop to counteract excessive accumulation of transcripts arising from genomic repeats in neurogenesis, in turn allowing maintenance of NSCs. However, the field of piRNA-mediated regulation of retrotranspositions in neuronal cells is still in its initial stages and confirmation of this hypothesis awaits formal validation. These studies have certainly the potential to identify not only biology of CNS- piRNA, but also offer the exciting possibility to explore the diagnostic and therapeutic values of these tiny RNA molecules in neurological disorders.

2 Rationale and Aim of the thesis

The mammalian hippocampus is one of the "niches" in which adult neural progenitor cells (aNPCs) persists throughout life [151]. Local generation of new neurons in the human hippocampus has been documented until old age and this process has implications for memory and age-related diseases, such as depression and Alzheimer's [152]. Adult neurogenesis is considered a form of structural plasticity of the brain. Indeed, environmental stimuli, such as enrichment and physical activity potentiate adult neurogenesis in rodents and this response is maintained until old age [153–159]. **The cellular and molecular mechanisms underlying homeostasis of adult neurogenesis and its response to environmental stimuli remain elusive.** Noncoding RNAs, such as miRNAs, and other epigenetic regulations are likely involved in this control [96,160].

The first question I will address in my thesis is built on preliminary evidence on the expression of piRNAs in somatic stem cells and their importance in stemness maintenance in the germline. In this view, the first aim of my work is to understand whether the Piwi-pathway plays any role in neural stem cells and in postnatal neurogenesis. One of the possible scenario is that, since the neuronal retrotransposition is an integral part of the neural development [150, 161], piRNAs might be highly active in the suppression of TEs in neurogenic aNPCs and in the regulation of other genes involved in neuronal development and differentiation processes.

The second question is to understand if Non-Coding RNAs (ncRNAs) have any role in environment dependent response of adult neurogenesis.

The rationale of this second part of my project is built on evidence that running stimulates hippocampal NPC proliferation and alters miRNA expression in rodents.

In particular, we selected miR-135a among a panel of miRNAs downregulated in running mice compared to resting ones. miR-135a was the only dysregulated miRNA which manipulation was able to reduce (overexpression) or increase (silencing) the proliferation of cultured aNPCs.

This, we hypothesized investigating miR-135a, which is involved in running induced neurogenesis, would allow the identification of the most prominent pathways that constrain NPC proliferative potential in the adult mouse hippocampus.

With this approach, we aim to uncover the proteins and pathways acting within this circuit-level context, hence providing a system-level biological understanding of scientific and therapeutic value.

Understanding the involvement of small ncRNAs in neurogenesis will provide better comprehension of the molecular regulations allowing homeostatic control of this process and its maintenance throughout life. This knowledge is a milestone toward the use of piRNAs and miRNAs as possible therapeutic agents or targets, to delay or prevent age-dependent loss of neurogenesis or pathological conditions arising from misregulation of neurogenesis.

Part II

Results and Discussion

3 The Piwi pathway

Data presented in this section have been submitted for publication:

"Mili function maintains hippocampal neurogenesis and implicate Piwi pathway in polyribosome assembly and translation control"

Gasperini C[1], Pelizzoli R[1], Lo Van A[1], Mangoni D[1], Cossu RM[1], Bianchini P[1], Pascarella G[2], Bielefeld P[3], Scarpato M[1], Pons-Espinal M[1], Diaspro A[1], Fitzsimons CP[3], Carninci P[2], Gustincich S[1] and De Pietri Tonelli D[1]

1. Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy
2. Division of Genomic Technologies, RIKEN Center for Life Science Technologies, Yokohama, Japan
3. Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands

Abstract

piRNAs are small, 26-32 nt single-stranded non coding RNAs that associate with P-element-induced wimpy testis (Piwi) proteins in various organisms. In mammals, piRNAs and piRNA-pathway proteins, are mostly thought to be restricted to germline cells, where they have been shown to repress transposable elements (TEs). Recent evidence however suggests that LINE1 is active in both neural stem cells (NSCs) and neural precursor cells (NPCs), thus playing a role of potential importance in genetic mosaicism and perhaps neuronal plasticity in mature neurons [166]. Despite this evidence, functions Piwi protein and piRNAs in the CNS are largely unknown.

Here, we find that Mili (Piwil2) and Mili-dependent piRNAs "the Piwi pathway" are abundantly expressed in neural progenitor cells (aNPCs) but depleted in differentiated progeny of the postnatal mouse hippocampus. Moreover, inhibition of the Piwi pathway impairs neurogenesis and increases generation of reactive glia, while induction of aNPCs reactivity reduces the Piwi pathway levels. Transcripts involved in ribosome assembly and translation are main targets of the Piwi pathway in aNPCs. These results identify essential functions of the Piwi pathway in brain, suggesting it controls protein synthesis in somatic stem cells.

This study represents a pioneering research on the putative role of Piwi proteins and piRNAs in neurogenesis and neuronal differentiation, and has the intrinsic potential to better understand piRNA-related mechanism in mammalian brain both in physiological and pathological conditions.

3.1 Hippocampal expression of Mili is restricted to neural progenitor cells (aNPC) and decreases in neurogenesis

To investigate expression of the Piwi pathway in brain, we compared the abundance of Miwi and Mili, the principal proteins required for piRNA production [167,168], in testis, whole hippocampus and primary cultures of hippocampal aNPCs from postnatal mice. As expected Miwi protein was very abundant in testis, but almost undetectable in hippocampus or aNPCs (Figure 3.1A). Mili protein was almost undetectable in the hippocampus, but surprisingly its expression in aNPCs was about 40 % of testis (Figure 3.1B) and >4 fold enriched compared to hippocampal neurons (Figure 3.1C).

To confirm this observation *in vivo*, we took advantage of a split-Cre viral approach [169] to label aNPCs and their progeny by Cre recombinase-dependent activation of fluorescent reporters [97, 170]. Five days post-injection (dpi) of split-Cre viruses in the dentate gyrus (DG) of 8-week-old Td-Tomato Cre-reporter mice, we found immunofluorescence staining for Mili in Td-Tomato+ (Td+) aNPCs in the SGZ of the DG (Figure 3.1D). Next, to quantify Mili expression in neurogenesis, we evaluated its transcript levels by quantitative Real-Time PCR (qRT-PCR) in sorted Td+ aNPCs and differentiated progeny upon split-Cre-injection in DG of Td-Tomato mice. Mili mRNA expression was abundant in Td+ aNPCs (Figure 3.1E, 10 dpi) compared to Td- cells and decreased in adult-born Td+ neurons (Figure 3.1E, 30 dpi).

To corroborate this observation at the protein level, we analyzed Mili protein abundance in cultures of aNPCs in proliferative media (Figure 3.1F, Days In Vitro (DIV) 0) and upon induction of neuronal differentiation with virally transmitted Achaete-Scute homolog 1 (Ascl1) expression vector [171] (Figure 3.1F, DIV 7-21), an approach that allows to obtain >90 % of neurons *in vitro* [97]. These experiments confirmed the enrichment of Mili in aNPCs and its decrease along with neurogenesis, at both RNA and protein levels (Figure 3.1F).

Figure 3.1: Next page - (**A**) Miwi and (**B**) Mili protein abundance in testis, hippocampus and cultured aNPCs from adult mice. (**C**) Mili protein abundance in cultured hippocampal neurons and aNPCs. (**D**) Representative images of Mili (green) expression in Td+ aNPC (red) in the hippocampal subgranular zone (SGZ); arrows indicate Td+ and Mili double-positive cells. (**E**) Mili mRNA expression in sorted Td+ and Td– cells at 10 or 30 days after in vivo transduction with split-Cre viruses in postnatal hippocampus. (**F**) Mili protein abundance in aNPCs (in proliferative media, 0 DIV) and 4-14 DIV upon induction of neurogenesis. Data are expressed as mean ±Standard Error of the Mean (SEM), n=3 independent experiments. t-student test or one-way ANOVA Bonferroni as post hoc: *$p<0.05$, **$p<0.01$, ****$p<0.0001$. GCL, granular cell layer; H, Hilus. Scale bar 10 µm.

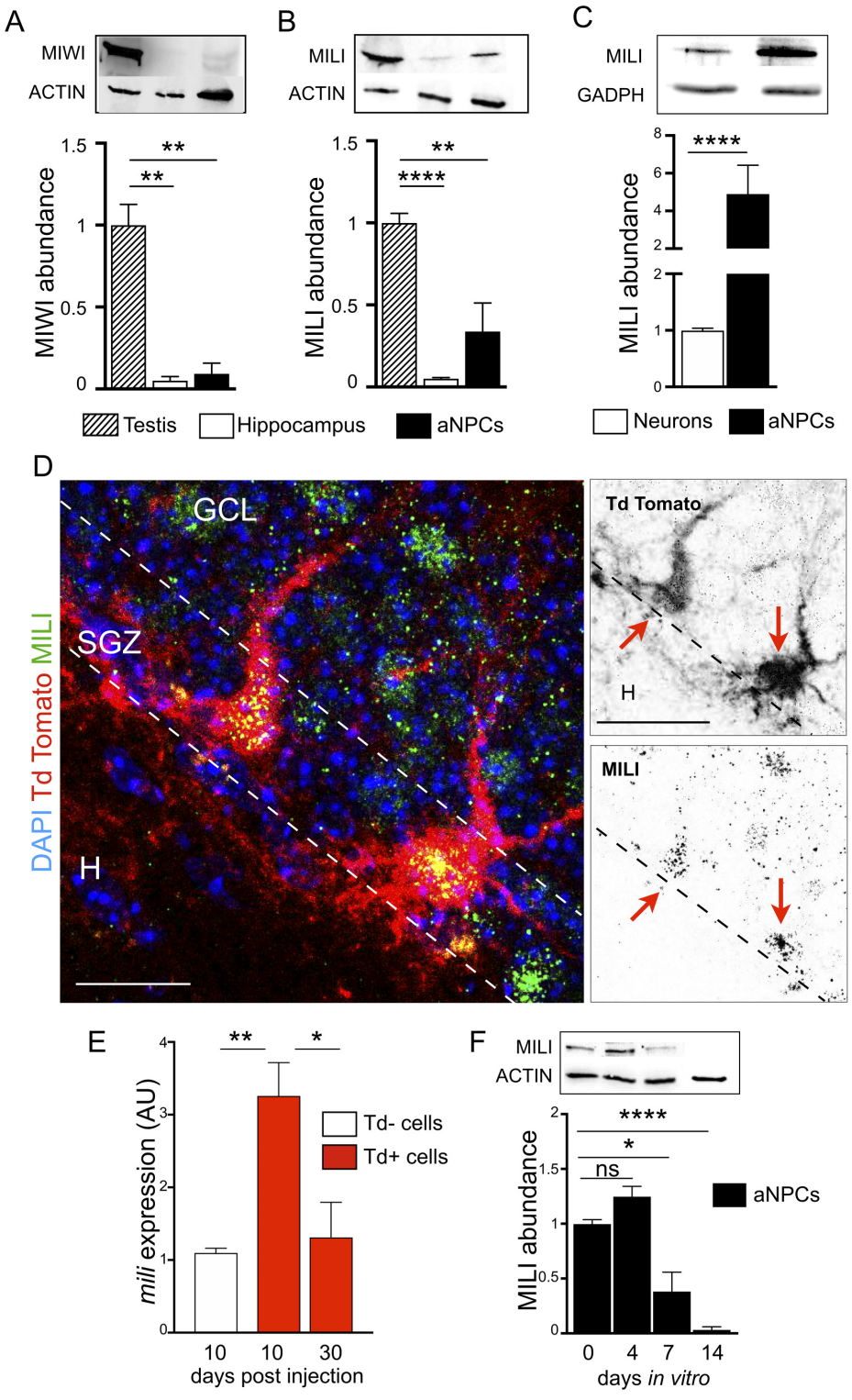


Figure 3.1: Hippocampal expression of Mili is restricted to neural progenitor cells (aNPC) and decreases in neurogenesis. Caption on previous page.

3.2 PiRNA clusters are dynamically expressed in neurogenesis and one is conserved in mouse and human

To ascertain whether enrichment of Mili in aNPCs correlates with piRNA expression, we performed small RNA sequencing (RNA-seq) from cultured aNPCs in proliferation (Figure 3.2A-C, DIV0) and at two time points upon induction of neuronal differentiation (Figure 3.2A-C, DIV4, 7). *Bona fide* piRNA were identified by alignment on piRBase [172], showed an average length of 30 nt (Figure 3.2A) and displayed U bias at the $5'$ end (Figure 3.2B), as previously reported [135]. These piRNAs clustered in 298 genomic locations (Figure 3.6A) and their level of expression was dynamic in neurogenesis, showing a transient peak upon onset of neurogenic differentiation (Figure 3.2C, DIV 4 and Supplementary Figure 3.6B).

We validated *in vivo* four of the most abundant piRNA clusters from the sequencing data in sorted Td+ cells, compared to Td- cells (Figure 3.2D) confirming the enrichment of piRNA in aNPC of the DG (Figure 3.2D). In line with this finding, expression of piRNA clusters in short RNA-seq from RIKEN FANTOM5 database [66] is enriched in human neural stem cells compared to astrocytes (Figure 3.6C-D). Remarkably, one of the most abundant piRNA cluster (hereafter referred as piR-cluster 1) in aNPCs and early-born neurons (Figure 3.2C), maps on mouse chromosome 8 (Chr8) and is conserved in human Chr16, overlapping with two glycine-Transfer RNA (tRNA) genes (Figure 3.2E).

Importantly, a piRNA encoded by the piR-cluster 1 (*i.e.*, piR-61648) was recently found to be enriched in human and murine somatic tissues but depleted in gonads [173], suggesting this cluster might be selectively expressed in somatic stem cells. We found that piR-cluster 1 is also expressed in human neural stem cells (Figure 3.6). These results suggest possible roles for the Piwi pathway in aNPCs maintenance and/or differentiation.

Figure 3.2: Next page - (**A**) Size distribution of piRNA reads. (**B**) U bias at the 5 end of piRNAs. (**C**) Mean expression of 298 piRNA clusters in aNPC (DIV0) and upon induction of neurogenesis (DIV4-7). Arrows indicate piRNA cluster 1 (piR-cluster 1). (**D**) Relative expression of four representative piRNA clusters in sorted Td+ and Td– cells 10 days post injection (dpi) of split-Cre viruses in postnatal hippocampus. (**E**) (left) Scheme representing genomic location of piR-cluster 1 in the mouse chromosome (Chr) 8 and human Chr 16; (Right) location of the piR-cluster 1 in the intron 1 of Vac14 gene in mouse and human and sequences of piR-cluster 1 (1A and 1B, underlined red text) inside tRNA-Gly genes (underlined black text). Data are expressed as mean ±SEM, n=2 (A-C) and n=3 (D) independent experiments. t-student test as post hoc: $*p<0.05$, $**p<0.01$.

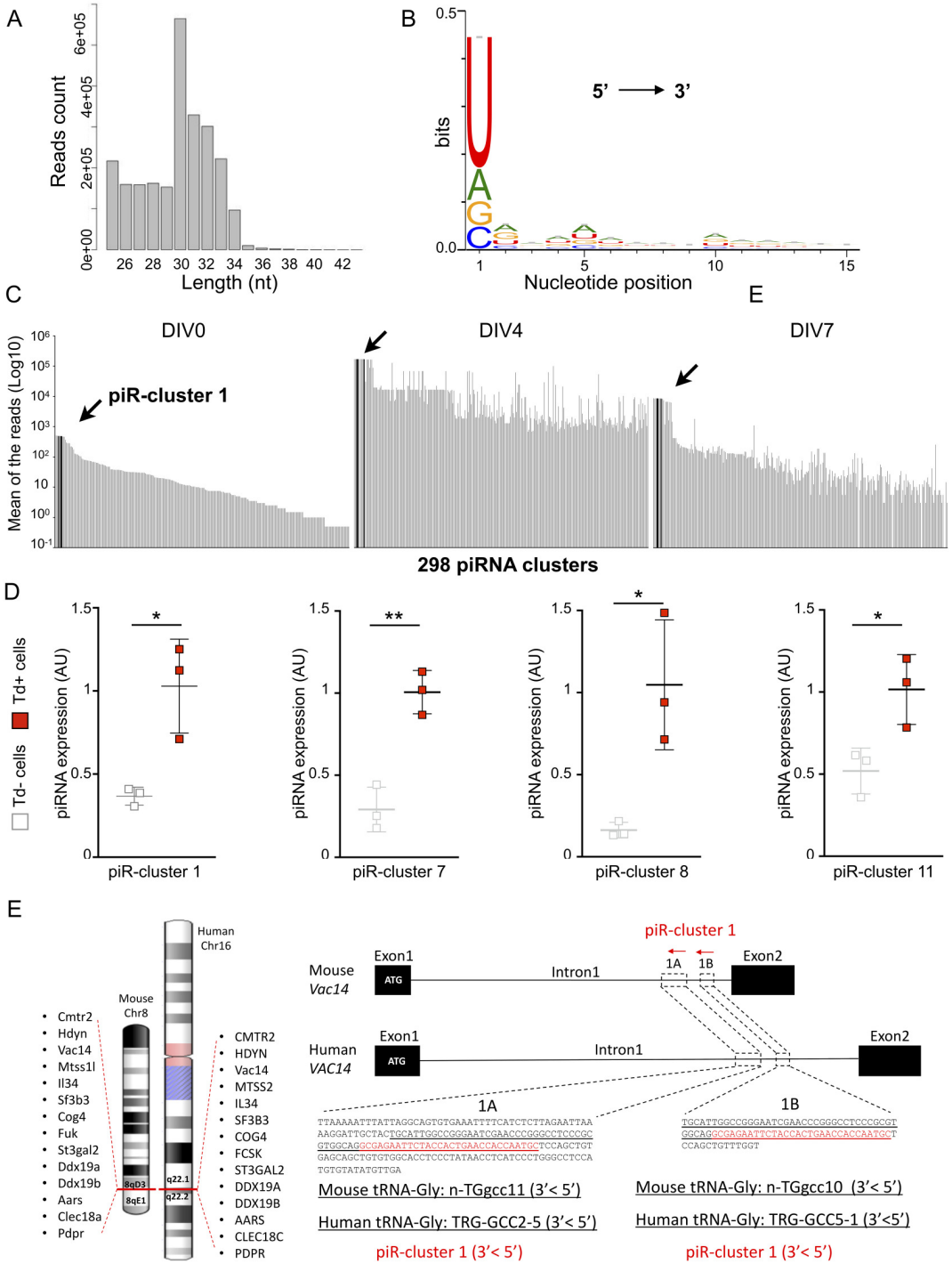


Figure 3.2: PiRNA clusters are dynamically expressed in neurogenesis and one is conserved in mouse and human. Caption on previous page.

3.3 The Piwi pathway sustains neurogenesis and mediates activation of aNPCs in the postnatal hippocampus

To infer functions of the Piwi pathway in neurogenesis, we silenced Mili in aNPCs by transducing *in vitro* a lentivirus transcribing a short-hairpin RNA targeted against mili transcript (shMili). As control, we used a lentivirus transcribing a scramble short-hairpin RNA (shControl). Both vectors encoded EGFP reporters to select virally transduced cells by FACS. Levels of both Mili transcript and protein were significantly reduced in the aNPCs with shMili, compared to shControl (Figure 3.3A).

To ascertain whether Mili KD in aNPCs led to piRNA depletion, we quantified by qRT-PCR four representative piRNAs from clusters abundantly expressed in the RNA-seq data; all four were significantly reduced compared to control aNPCs (Figure 3.3B). Miwi transcript levels remained low in Mili KD aNPCs, allowing to exclude potential compensatory effects on piRNA production due to Miwi increase in these cells (Figure 3.7). Importantly, these results confirmed the enrichment of Mili (Figure 3.1) as well as the identity of the RNA-seq reads as Mili-dependent piRNAs (Figure 3.2), in aNPCs.

Next, we cultured aNPCs expressing shMili or shControl in differentiation media, a condition that allows to obtain astrocytes and neurons in equal proportions [97]. Mili KD led to a dramatic increase of Glial fibrillary acidic protein (GFAP) at both mRNA and protein levels, compared to control cells (Figure 3.3C). Increased GFAP levels are generally regarded as hallmark of reactive glia [174, 175]. To investigate whether Mili KD may led to astrocyte reactivity, we injected bilaterally a synthetic oligonucleotide antisense to Mili (GapmeR,

Figure 3.3: Next page - (**A**) Mili mRNA (left) and protein (right) levels in aNPCs treated with a scrambled shRNA (Control) or antisense to Mili (Mili KD). (**B**) Relative expression levels of four sequences highly represented in piRNA clusters in control and Mili KD aNPCs. (**C**) Gfap mRNA (left) and protein (right) levels in control and Mili KD aNPCs upon onset of differentiation. (**D**) Schematic representation of the in vivo experiment. (**E**) Mili mRNA (left), protein (middle) and Gfap mRNA (right) levels in dissected DG of mice injected with scrambled (Control) or anti-Mili GapmeR (Mili KD). (**F**) Representative images of a brain slice stained with anti-GFAP antibody from a postnatal mouse 30 days post injection (dpi) of scrambled (Control, left hemisphere), or anti-Mili GapmeR (Mili KD, right hemisphere) in the DG. (**G**) (Left panels) Representative micrograph showing immunostaining for GFAP (green), BromodeoxyUridine (BrdU) (red), NeuN (white) and nuclear DNA (blue) in the hippocampal SGZ of mice 30 dpi with scrambled (control) or anti-Mili GapmeR (Mili KD); (Right Panels) quantification of the percentage of BrdU and NeuN (white arrowheads), or BrdU and GFAP (yellow arrowheads), double-positive cells over total BrdU+ cells in hippocampal SGZ. (**H**) Relative expression of mRNAs (indicated) markers of reactive astrocytes in DG from mice injected with Scrambled (Control) or anti Mili (Mili KD) GapmeRs. (**I**) Mili mRNA (left) and piR-cluster 1 (right) relative expression in sorted aNPCs from Nestin-GFP mice treated with Saline (Control) or Kainic Acid (KA)). Data are expressed as mean ±SEM, n=3 independent experiments (*in vitro*); n=5 (E); n=7 (F, G); n=3 (H, I). t-student test as post hoc: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bars: 1 mm (F); 100 µm (G).

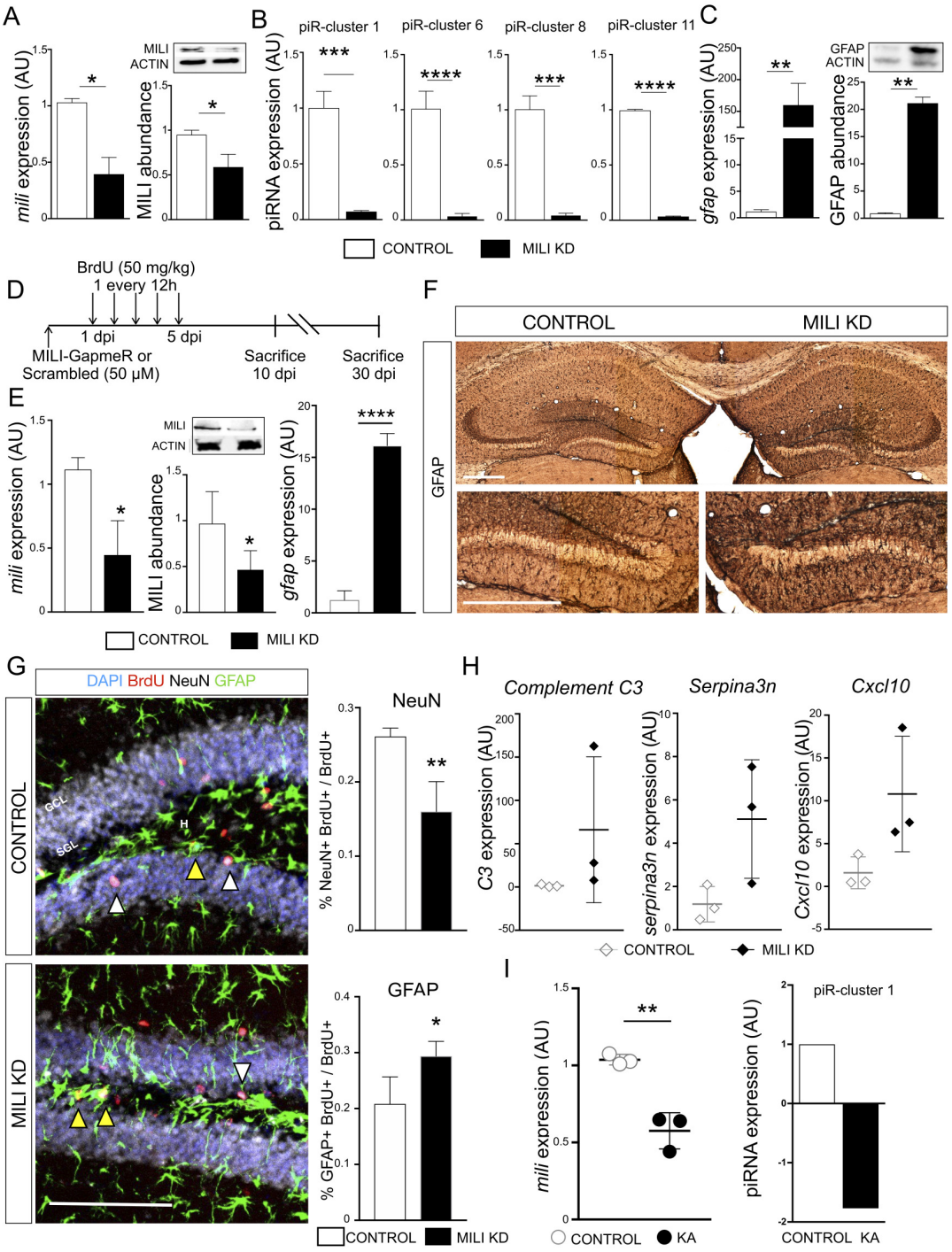


Figure 3.3: The Piwi pathway sustains neurogenesis and mediates activation of aNPCs in the postnatal hippocampus. Caption on previous page.

Mili KD), or a scrambled GapmeR (control) in DG of 6 to 8 weeks old mice (Figure 3.3D). 48 hours after, Mili was significantly reduced in DGs injected with GapmeR antisense to Mili, at both transcript and protein levels compared to control DGs (Figure 3.3E). In the same samples, we found an increase in Gfap mRNA expression upon Mili KD compared to controls (Figure 3.3E), confirming *in vitro* results.

Moreover, immunohistochemistry analysis of brain sections 30 days after GapmeRs injection revealed a marked increase in GFAP staining in the ipsilateral hippocampus injected with GapmeR antisense to Mili, compared to the contralateral one injected with the control GapmeR (Figure 3.3F). To ascertain whether Mili KD may lead to generation of reactive glia, in a second cohort of mice immediately after GapmeR injections, we administered BrdU to label dividing cells (Figure 3.3D). 30 days after GapmeR injection, we quantified the fate of BrdU-labeled cells and found that Mili KD resulted in an increased proportion of adult-born GFAP/BrdU double-positive astrocytes (Figure 3.3G, H) and to a decreased proportion of NeuN/BrdU double-positive neurons, compared to control DG (Figure 3.3G, H). These results indicate that depletion of Mili in postnatal DG, repress neurogenesis and result suggests aberrant generation of astrocytes. Indeed, levels of reactive astrocyte markers complement component C3, Serpina3n and Cxcl10 [174, 175] were increased in the DG upon Mili KD, compared to control (Figure 3.3H), suggesting that Mili KD increases astrocyte reactivity.

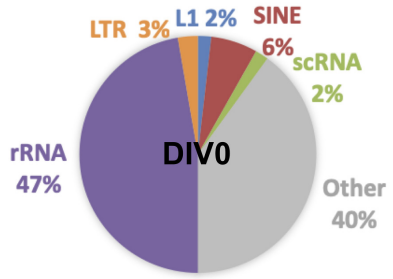
To further address this possibility, we administered KA in postnatal mice carrying a GFP knock-in allele in the gene encoding the neural stem and progenitor cells marker Nestin [176], a treatment that induces neuronal hyperexcitation similar to epilepsy and known to accelerate conversion of hippocampal adult neural stem cells into reactive astrocytes [170]. 3 days after KA administration, we found a significant decrease of Mili mRNA in sorted cells from Nestin-GFP mice treated with KA compared to saline (Control) and a concurrent decrease in the expression of piR-cluster 1 (Figure 3.3I). Altogether, these results indicate that the Piwi pathway sustains neurogenesis and suggest it is involved in preventing reactive gliosis in the postnatal hippocampus.

3.4 *In silico* piRNA target analysis predicts noncoding RNAs and mRNAs involved in translation

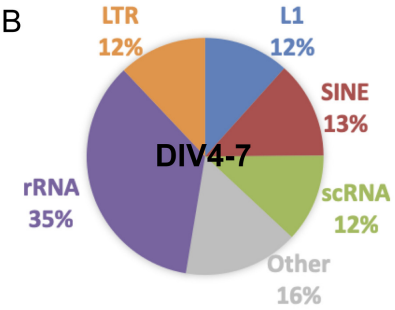
In order to identify possible targets of Mili-dependent piRNAs in neurogenesis, we did in silico analysis [135] of the piRNAs expressed in proliferating (Figure 3.4, DIV 0) and upon induction of neuronal differentiation (Figure 3.4, DIV 4-7). In gonads, piRNAs repress mainly TEs but also other genic RNAs [167].

Hence, we aligned piRNAs to both noncoding RNA (Figure 3.4A, B) and mRNAs (Figure 3.4C).

A



B



C

mRNA targets DIV0
Btn1a1
Elf1
Hdgfl2
Kitl
Malat1
Rn5s
Tmtc1
Trim2
Vac14

mRNA targets DIV4-7		
Aqr	Il27	**Rpl3**
Arhgap12	Il31ra	**Rpl7a**
Arrb1	Impg1	Rsph3a
Atf2	Itga9	Rsph3b
BC107364	Jarid2	Ryr3
Brip1os	Lama2	Scaper
Camk1d	**Lars2**	Slc22a22
Cntn3	Luzp1	Slc4a8
Cntn5	Malat1	Snhg1
Col6a6	Mkl2	Snhg12
Cyp2b9	Mmd2	Snhg5
Dcaf10	Naaladl2	Spata5
Dnah7b	Nlrp4a	Spryd7
Eif4a2	Nop56	Tor1aip2
Enox2	Platr30	Trim58
Fam129a	**Pum1**	Trpm3
Gas5	**Rack1**	Vwa3a
Gphn	**Rpl13a**	Yipf1
Ica1	**Rpl17**	Zfas1

GO biologica process DIV4-7		Proteins involved in the biologial process
Term	p value	
Translation	0,00031	Eif4a2, Lars2, Rack1, Rpl13a, Rpl17, Rpl3, Rpl7a
Regulation of translation	0,0028	Fam129a, Pum1, Rack1, Rpl13a

KEGG pathways DIV4-7		Proteins involved in the biologial process
Term	p value	
Ribosome	0,0059	Rpl13a, Rpl17, Rpl3, Rpl7a

ID ENSEMBL	FULL NAME
Eif4a	eukaryotic translation initiation factor 4A2
Lars2	leucyl-tRNA synthetase, mitochondrial
Rack1	receptor for activated C kinase 1
Rpl	ribosomal protein L
Fam129a	family with sequence similarity 129, member A
Pum1	pumilio RNA-binding family member 1

Figure 3.4: *In silico* piRNA target analysis predicts noncoding RNAs and mRNAs involved in translation. (**A**, **B**) Pie plots showing proportions of noncoding RNAs that are predicted piRNA targets in aNPCs (DIV0) and upon induction of neurogenesis cells (DIV4-7). (**C**) Tables summarizing genic RNAs predicted piRNA targets in aNPCs (DIV0) or upon induction of neurogenesis (DIV4-7); Gene Ontology (GO) biological process and Kyoto Encyclopedia of genes and genomes (KEGG) pathway analysis of genic piRNA targets, ID ENSEMBL and Full names of genic targets involved in translation control are indicated.

Surprisingly, Ribosomal RNAs (rRNAs), especially rRNA 5S, are main ncRNAs predicted targets of piRNAs in proliferating aNPCs (47 %, Figure 3.4A). Other small ncRNAs, in particular tRNAs, are also very represented (40 %, Figure 3.4A). This finding corroborates previous evidence in other somatic tissues, but not brain, indicating that the majority of small RNAs associated to the Piwi protein HIWI2 are tRNA-derived piRNAs [177]. In contrast, the percentage of predicted piRNA targets corresponding to TEs (here comprising endogenous retroviruses long terminal repeats (LTR); L1s and SINEs increases in differentiating cells (37 %, Figure 3.4B), at the expense of rRNAs and other small RNAs but their percentage remains relevant (51 %, Figure 3.4B).

Given that observed increase of active L1s in neurogenesis [141, 150], this finding would be compatible with a higher proportion of TEs-derived piRNAs in

differentiating aNPCs. Analysis of mRNA targets indicated a set of genes encoding proteins involved in translation, such as four ribosomal proteins RpLs and the leucyl-tRNA synthetase Lars2 (Figure 3.4C). GO and KEGG analysis confirmed a prevalence of "translation" and "ribosome" pathways as main biological processes. This analysis provides a new insight on Mili-dependent piRNAs in aNPCs, suggesting a possible role of the Piwi pathway in the regulation of translation in neurogenesis.

3.5 Depletion of Mili and Mili-dependent piRNAs enhances polysome assembly in aNPCs

To validate piRNA targets (Figure 3.4) we quantified expression of some ncRNA and mRNA upon Mili KD (Figure 3.5A, B). Mili KD significantly elevated levels of five ncRNAs (LINE1, SINE B1, rRNA 5S) and mRNAs (Lars2, Rpl13a, Rpl17a) in aNPCs and neurogenesis (Figure 3.5A) or in proliferating aNPCs (Figure 3.5B) compared to control cells, validating them as Mili-dependent piRNA targets.

To gain insight into a possible mechanism of the Piwi pathway in neurogenesis, we quantified the abundance of total RNA and protein extracted from proliferating aNPCs upon Mili KD (Figure 3.5C). Total RNA content normalized on the number of cells, was not significantly altered between control and Mili KD cells. However, the majority of samples from Mili KD cells had an increased, yet not statistically significant, amount of soluble proteins compared to control (Figure 3.5C). Increased association of ribosomes on mRNAs (*i.e.*, polysomes) is generally regarded as a hallmark of active translation. Therefore, we quantified the polysome assembly in proliferating aNPCs upon Mili KD by means of STED Nanoscopy (Figure 3.5D-E), an approach that resolves polysomes but not 40S, 60S ribosome subunits or monosomes [178]. Remarkably, Mili KD dramatically increased polysome assembly, as revealed by immunostaining for the RPL 26, compared to control aNPCs (Figure 3.5D). In particular occupancy, concentration and size average of the particles were increased (Figure 3.5E). To corroborate these findings we evaluated growth, proliferation and stemness of Mili KD aNPCs in proliferative media and found no differences in any of these parameters compared with control cells (Figure 3.7). These results support our hypothesis that the Piwi pathway modulates protein synthesis in neurogenesis.

3.6 Discussion

To the best of our knowledge, this is the first report of a functional a role for Mili and Mili-dependent piRNAs in the mammalian brain. The enrichment of Mili and piRNAs in aNPCs and the identification of a piRNA cluster conserved in mouse and human constitute a resource for future studies in brain. Specifically,

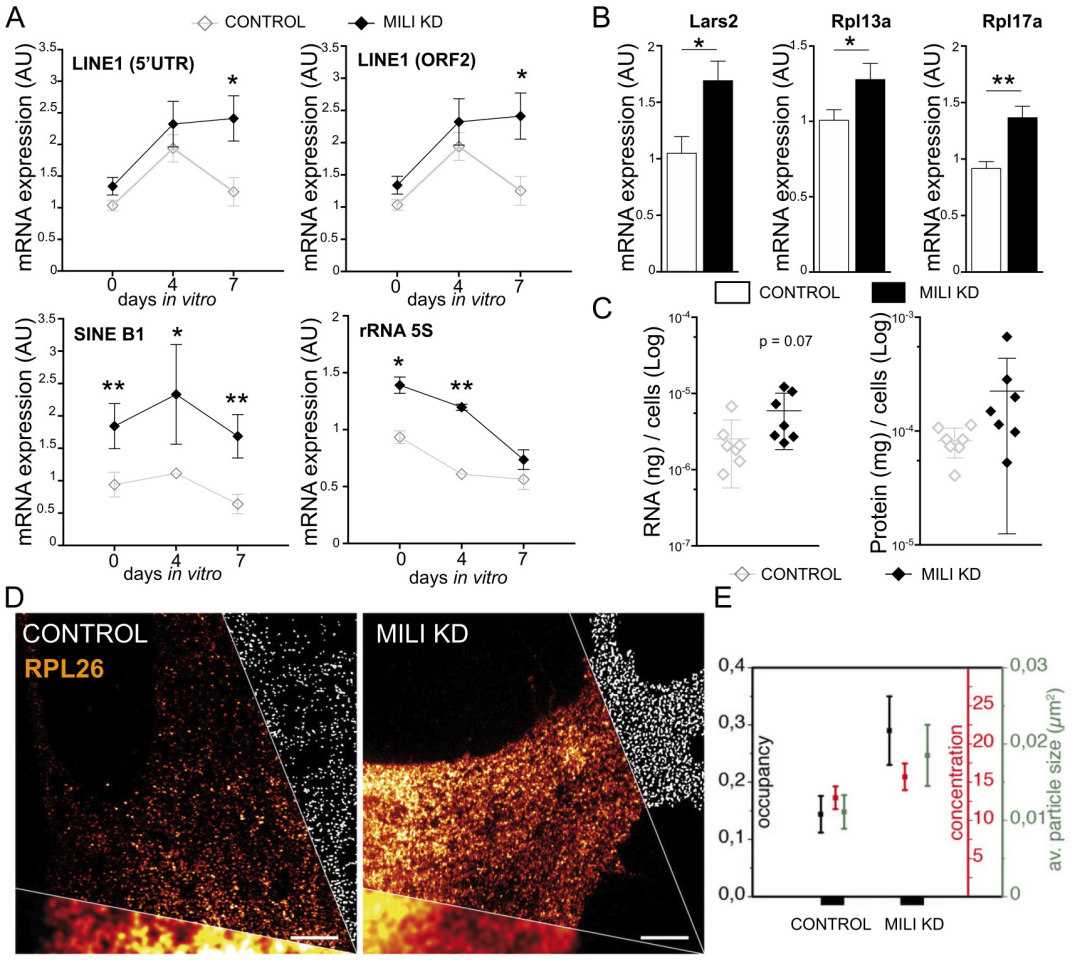


Figure 3.5: Depletion of Mili and Mili-dependent piRNAs enhances and polysome assembly in aNPCs. (**A**) qRT-PCR validation of main classes (indicated) of noncoding RNA targets of piRNAs in aNPCs (DIV0) and in neurogenesis (DIV4-7) upon treatment with a scrambled shRNA (Control) or antisense to Mili (Mili KD). (**B**) qRT-PCR validation of three representative mRNA targets of piRNAs in neurogenesis upon treatment with a scrambled shRNA (Control) or antisense to Mili (Mili KD). (**C**) RNA and protein abundance relative to cell number in control or Mili KD aNPCs. (**D**) Representative micrograph showing magnification of aNPCs cytoplasm immunolabeled with ATTO-488 against Ribosomal Protein (RPL)26 and imaged with g-STED nanoscopy (middle cut). In the additional cuts, confocal microscopy (bottom) and thresholding for the analysis (top). Right panel: control and Mili KD aNPCs are compared plotting the normalized distributions of the occupancy, concentration and average of particle size of each polyribosome particle. Scale Bar, 2 µm. Data are expressed as mean ±SEM, n=3 (7 in C) independent experiments. t-student test as post hoc: $*p<0.05$, $**p<0.01$.

here we find that Mili sustains aNPCs neurogenic differentiation and modulates glial reactivity, indicating that the Piwi pathway is required for hippocampal neurogenesis. Mechanism of this regulation likely involves control of protein translation.

Our study provides an unanticipated new role of the Piwi pathway in the regulation of protein synthesis in neural stem cells. A tight balance between quiescence, activation and differentiation is required for lifelong maintenance of neural stem cells. The switch between quiescent and activated state crucially depends on protein synthesis rate [179], while low translation maintains somatic stem cells in undifferentiated state [180].

In 2008, Sampath et al. found that global translation was low in undifferentiated embryonic stem cells compared to embryonic body and that differentiation induced an anabolic switch. The increase in translation in differentiated cells coincides with a significant increase in the content of total RNA ($\sim 50\,\%$), ribosomal RNA ($\sim 20\,\%$), and proteins ($\sim 30\,\%$). Remarkably, differentiation increases polysome density compared to undifferentiated embryonic stem cells [181]. in 2016, Blanco et al. showed that also skin stem cells have lower protein synthesis than committed cells and that low translation functionally contributes to their maintenance [182]. More recently, it has been observed that low protein synthesis rate in stem cells associates with their low cellular metabolism. Activation for proliferation and commitment to differentiate requires a huge remodeling of cellular metabolism leading to substantial variations in energy production and consumption, which correlates with changes in the protein synthesis rate [179].

In fact, during steady state, transcriptional control is the main determinant of the cellular proteome, whereas during early stages of state transition (such as differentiation), translational control becomes the major determinant [183]. Translational control allows cells to promptly respond to internal and external stimuli, even before a new transcription program starts [184]. In this view, neurogenesis might also be controlled by protein synthesis rates, since it involves transition of NPCs through multiple stages and requires adaptation to the changing microenvironment, including metabolic switch.

At the mechanism level, Ribosomes are the center of the whole protein synthesis machinery and key for fine-tuning the proteome. Under physiological condition, ribosome abundance is not considered a limiting factor for translation initiation in stem cells, however, studies in Drosophila and mammals suggest that differentiation of stem cells relies on increased ribosomal biogenesis. In their study, Ingolia et al. concluded that an increased expression of ribosomal proteins at early stages of differentiation is required to boost the rate of global translation, observed at later stages [183].

Consistent with these findings, here we showed that aberrant activation of aNPCs and reduced neurogenesis caused by Mili KD, correlates with an increased expression of ribosomal biogenesis protein. In particular, we found the increased expression of ribosomal RNA subunit 5s and mRNA encoding ribosomal proteins L (RPL), Rpl3a, Rpl17 and Rpl26. These data suggest a role of the Piwi-pathway in the regulation of translation machinery and, possibly, protein synthesis. In addition to ribosomes, it is very likely that additional translational factors contribute to translational control in stem cells. The initiation factors eIFs are responsible for translation initiation, however there are only few studies on their role in stem cells. It has been reported that lack of eIFs in mouse is often embryonic or perinatal lethal and has detrimental effects on stem cells and normal development (reviewed in [180]). Interestingly, here we found that eIF4A, which is required for the binding of the 40S ribosomal subunits to the cap-complex of the mRNA, is one of the predicted piRNA targets in differentiating aNPCs. Among the predicted piRNA targets in aNPCs we found also tRNAs and TEs. Interestingly, tRNA fragments control translation in stem cells [185] and TEs (*i.e.*, L1s) regulate differentiation of adult NPCs [141, 150]. Together, these results strongly suggest an involvement of the Piwi pathway in the regulation of translation machinery.

Interestingly, tRNA-fragments control translation in stem cells [185] and TEs (*i.e.*, L1s) regulate differentiation of adult NPCs [141, 150].

The study of piRNAs in neural stem cells and in mature neurons represents a pioneering research on the potential role of these sncRNAs in neurogenesis and cell differentiation: here, we speculate that the Piwi pathway may sustain neurogenesis by simultaneous modulation of different RNAs involved in control of protein synthesis and differentiation. This evidence could open new insight toward a better understanding of brain ageing and age-related pathological conditions.

3.7 Supplementary informations

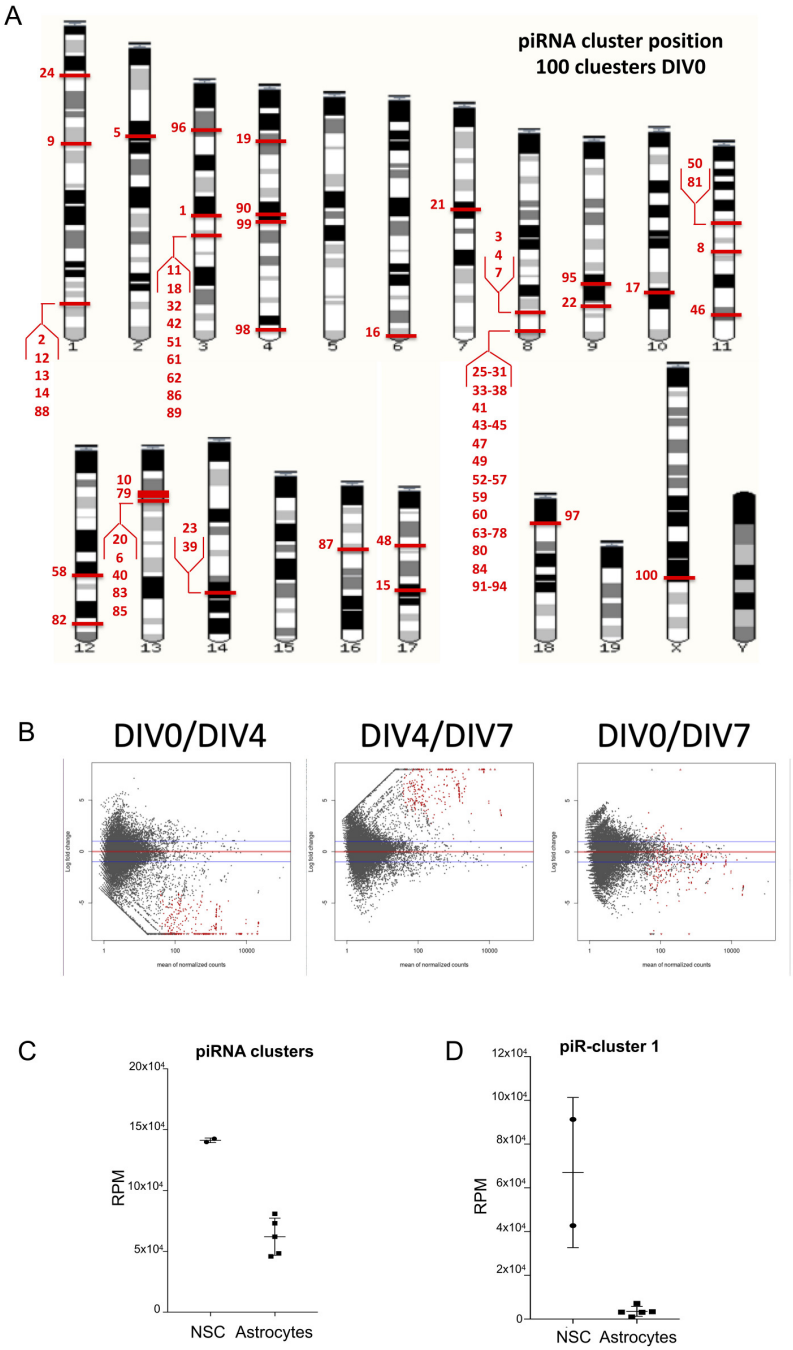


Figure 3.6: Genomic distribution and expression of piRNA clusters. (**A**) Genomic locations of 100 piRNA clusters expressed in proliferating aNPCs. (**B**) Pairwise comparison of 298 piRNA clusters differentially expressed in aNPCs (DIV0) or upon induction of neurogenesis (DIV4-7). (**C**) Relative abundance of piRNA clusters identified in Neural stem cells (NSC) or astrocytes. (**D**) Relative abundance of piR-cluster1 in NSC and astrocytes RPM: Reads per million.

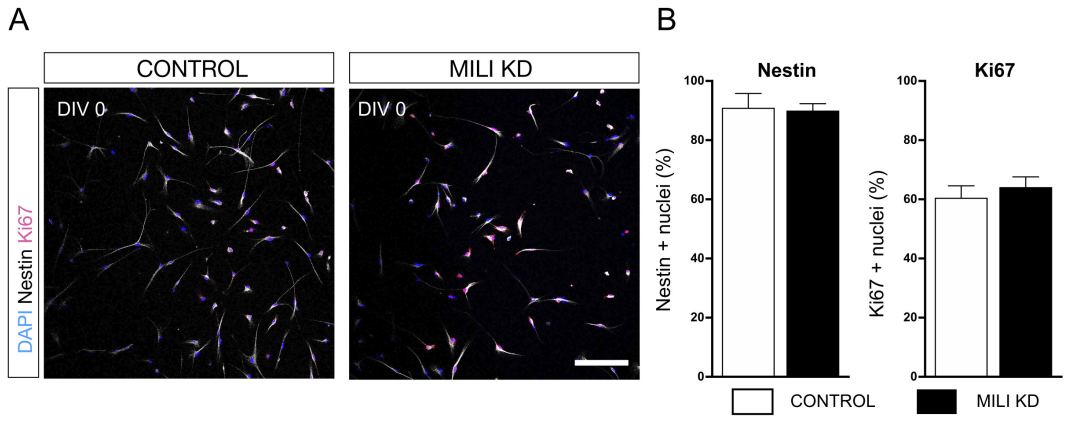


Figure 3.7: Mili KD does not affect aNPC proliferation and stemness. (**A**) Representative images of proliferating aNPC treated with lentivirus for a scrambled shRNA (Control) or antisense to Mili (MILI KD), stained with Nestin (white), Ki67 (purple), and nuclear DNA (blue). (**B**) Quantification of the percentage of Nestin or Ki67 positive cells over total cells. Data are expressed as mean ±SEM, n=3 independent experiments. Scale bar 50 µm.

4 Exercise-dependent microRNAs

Data presented in this section have been published in Stem Cell Reports in 2019:

"MiR-135a-5p Is Critical for Exercise-Induced Adult Neurogenesis"

Pons-Espinal M[1*], Gasperini C[1*],Marzi MJ[2], Braccia C[3], Armirotti A[3], Pözsch A[4,5], Walker TL[4,5], Fabel K[4,5], Nicassio F[2], Kempermann G[4,5] and De Pietri Tonelli D[1]

1. Neurobiology of miRNA, Istituto Italiano di Tecnologia (IIT), Genoa, Italy
2. Center for Genomic Science of IIT@SEMM, Istituto Italiano di Tecnologia (IIT), Milan, Italy
3. Analytical Chemistry Facility, Istituto Italiano di Tecnologia (IIT), Genoa, Italy
4. German Center for Neurodegenerative Diseases (DZNE) Dresden, Dresden, Germany
5. CRTD – Center for Regenerative Therapies, Technische Universitat Dresden, Dresden, Germany *Co-first authors

Abstract

Physical exercise stimulates adult hippocampal neurogenesis in mammals, and is considered a relevant strategy for preventing age-related cognitive decline in aging humans. However, its mechanism is controversial.

Here, by investigating miRNAs and their downstream pathways, we uncover that downregulation of miR-135a-5p mediates exercise-induced proliferation of adult NPCs in adult neurogenesis in the mouse hippocampus, likely by activation of phosphatidylinositol (IP3) signaling. Specifically, while overexpression of miR-135 prevents exercise-induced proliferation in the adult mouse hippocampus *in vivo* and in NPCs *in vitro*, its inhibition activates NPCs proliferation in resting and aged mice. Label free proteomics and bioinformatics analysis identifies 11 potential targets of miR-135 in NPCs, several of them involved in phosphatidylinositol signaling. Thus, miR-135a is key in mediating exercise-induced adult neurogenesis and opens intriguing perspectives toward the therapeutic exploitation of miR-135 to delay or prevent pathological brain ageing.

We hypothesize that exploiting these mechanisms is relevant for preventing age-related cognitive decline in humans and that our animal models can contribute to providing evidence-based recommendations for an active lifestyle for successful aging.

4.1 Running-induced proliferation downregulates miRNA expression in Nestin-positive adult hippocampal NPCs *in vivo*

To identify whether running alters miRNAs expression in adult hippocampal neural precursor cells (NPCs), six-week-old mice, expressing the fluorescent protein Cyan Fluorescent Protein /nuclear (CFP/CFPnuc) under the control of the regulatory elements of the Nestin gene (Nestin-CFP/CFPnuc), were housed under standard conditions, or equipped with a running wheel for 10 days (Figure 4.1A). Consistent with previous data, we found in the hippocampal SGZ of runner mice a significant increase in the number of BrdU positive cells (Figure 4.1B; p=0.005) and of BrdU/Nestin-CFP/CFPnuc double-positive NPCs (Figure 4.1D; p=0.007), also leading to an increased total number of Nestin-CFP/CFPnuc-positive NPCs (Figure 4.1C; p=0.136), suggesting an expansion of the proliferative NPCs pool.

We performed expression profiling of miRNAs in sorted Nestin-CFP/CFPnuc positive NPCs from the DG of resting and running mice, by means of TaqMan Low Density Array (TLDA) (three independent biological replicates, each containing a pool of Nestin-CFPnuc positive cells isolated from 8 mice per condition) and found 8 miRNAs that were reproducibly down-regulated in each replicate sample from runners, compared to resting mice (Figure 4.8). Of relevance, none of the miRNA identified in the TLDA were reproducibly induced in Nestin-CFP/CFPnuc positive NPC upon running (not shown). The three most downregulated miRNAs in Nestin-CFPnuc positive NPCs from the DG of running mice were miR-135a (mmu-miR-135a-5p), miR-190 (mmu-miR-190-5p) and miR-203 (mmu-miR-203-3p) (Figure 4.1E). Next, we examined by *in situ* hybridization [186, 187], the expression patterns of these three miRNAs in the DG of adult C57BL/6J mice housed in standard cages. As control for ISH we used a probe antisense to miR-9, a brain enriched miRNA known to be highly expressed in NPCs and neurons in embryonic and adult mice [188]. We found that miR-135a, miR-203 and miR-190 were expressed in the DG of adult mice, but only miR-135a and miR-190 were preferentially enriched in the SGZ, where NPCs are localized *in vivo* (Figure 4.1F).

Together, these results indicate that running decreases the expression of miRNAs in hippocampal aNPCs *in vivo*, opening the possibility that these miRNAs might be involved in the mechanism underlying running-induced proliferation of adult NPCs.

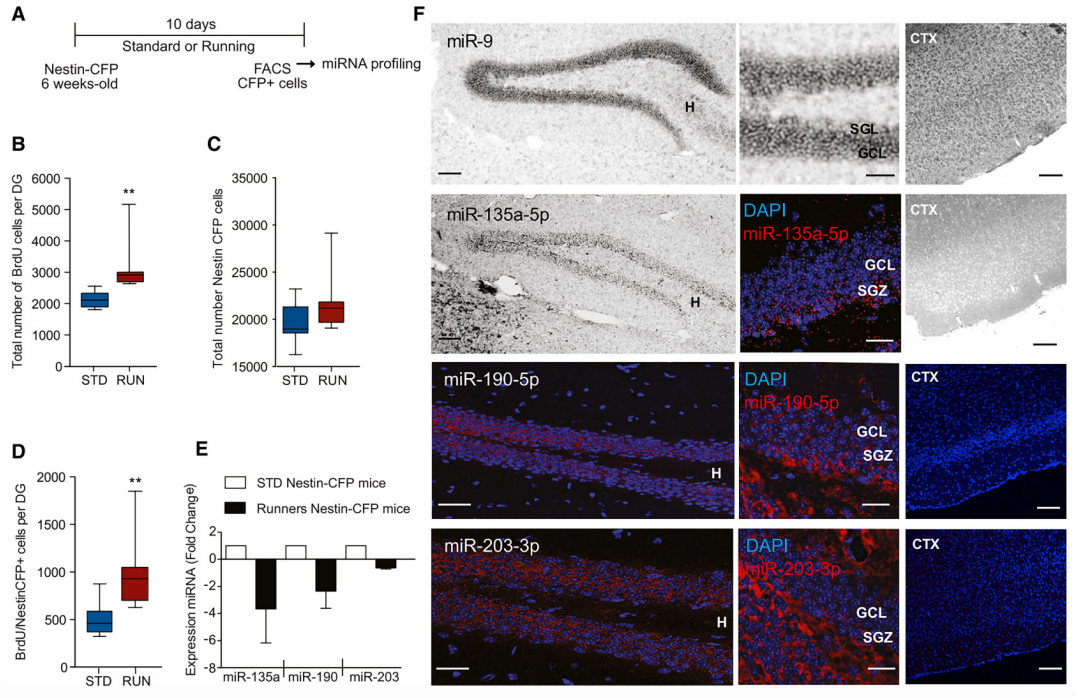


Figure 4.1: Running-Induced Proliferation Downregulates miRNA Expression in Nestin-Positive Adult Hippocampal NPCs *In Vivo*. (**A**) Schematic representation of the experiment. (**B**–**D**) (**B**) Total number of BrdU and (**C**) Nestin-CFP/CFPnuc positive adult neural progenitor cells (NPCs), or (**D**) proportion of BrdU/Nestin-CFP/CFPnuc double-positive cells counted from the hippocampal SGZ of Nestin-CFP/CFPnuc mice under standard (STD) or running (RUN) conditions for 10 days; n=8 mice per group. (**E**) Quantification of relative expression levels of miR-135-5p, miR-190-5p, and miR-203-3p by TLDA in sorted Nestin-CFP/CFPnuc NPCs from the hippocampus of adult mice in STD or RUN conditions. (**F**) Representative micrographs showing expression of miR-9-5p (positive control), miR-135-5p, miR-190-5p, and miR-203-3p by in situ hybridization in the DG and cortex (CTX) of 6-week-old C57Bl6J mice. H, hilus, GCL, granular cell layer; SGZ, subgranular zone. Data are expressed as means ±SEM, n=3 independent experiments. One-way ANOVA Bonferroni as post hoc: $^{**}p<0.01$. Scale bar, 100 mm (large panel), 50 µm (small panel).

4.2 miR-135a inhibits cell cycle progression of cultured adult NPCs

To investigate this possibility, we compared expression of the 3 miRNAs in cultures of primary hippocampal NPCs [189] in quiescence and proliferative conditions. Quiescence is here operationally defined as "non-proliferative" and induced *in vitro* by the addition of Bone Morphogenetic Protein (BMP4) [190] to the culture medium containing Fibroblast Growth Factor 2 (FGF2/bFGF). Proliferation media was supplemented with both FGF2 and Epidermal Growth Factor (EGF). As expected, proportion of BrdU-positive NPCs in proliferative media was higher than in quiescence media (Figure 4.2A-B; $p<0.001$) and, consistent with miRNA profiling of running mice (Figure 4.1), proliferating NPCs had significantly lower levels of miR-135a, miR-190 and miR-203 compared to

cells in quiescence (Figure 4.2C $p<0.001$).

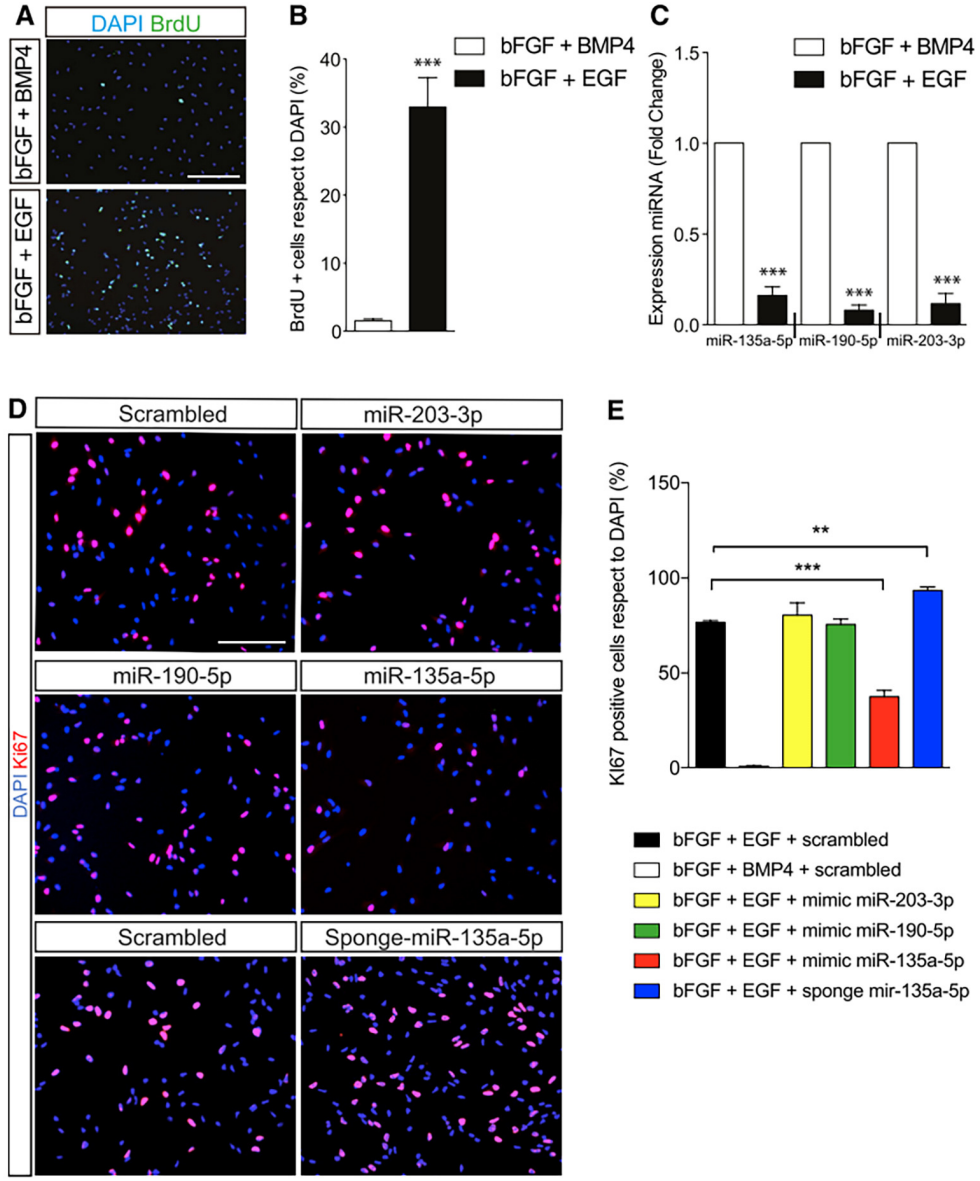


Figure 4.2: miR-135a Levels in Adult NPCs Are Cell-Cycle Dependent and Its Modulation Affects Cell Proliferation *In Vitro*. (**A** and **B**) Representative micrographs showing (**A**) BrdU-positive and (**B**) quantification in primary hippocampal adult NPCs cultured in proliferative medium (EGF + bFGF), or quies- cence medium (BMP4 + bFGF). (**C**) Relative miRNA fold change expression. (**D** and **E**) Representativemicrographs showing (**D**) Ki67-positive NPCs and (**E**) quantification cultured in proliferative medium (EGF + bFGF) upon transfection with 50 nM scrambled con- trol, miR-203-3p, miR-190-5p, miR-135a-5p mimics, or upon transduction with lentivirus transcribing a sponge for miR-135a (sponge miR-135a, *i.e.*, loss of function) or virus expressing control (scrambled) RNAs. Data are expressed as means ±SEM, n=3 independent experiments containing three replicates. One-way ANOVA Bonferroni as post hoc. $^{**}p<0.01$, $^{***}p<0.001$. Scale bars, 50 µm.

To ascertain whether miR-135a, miR-190 or miR-203 affect adult NPC proliferation, we transfected synthetic miRNA mimics or scrambled control into NPCs *in vitro* and quantified the proliferative marker Ki67 [191]. Overexpression of miR-135a, but neither miR-203, nor miR-190 was sufficient to reduce the proliferation of NPCs (Figure 4.2D-E; $p<0.001$). Conversely, inhibition of miR-135a, upon transduction with a virus expressing a sponge (see below and Figure 4.3A), led to a significant increase in NPC proliferation (Figure 4.2D-E). These results indicate an anti-proliferative function of miR-135a in adult hippocampal NPCs *in vitro*.

4.3 Validation of lentiviruses to overexpress / downregulate miR-135a in NPCs

To confirm this result *in vivo*, we transduced lentiviruses expressing either a short-hairpin precursor of miR-135a (sh-miR-135, gain of function), or a sponge for miR-135a (loss of function), or scrambled control RNA sequences in NPC cultures (Figure 4.3A). Expression of miR-135a in NPCs was higher upon transduction with sh-miR-135a and significantly reduced upon transduction with sponge for miR-135a, compared to controls (Figure 4.3A).

We injected the miR-135a sponge or a scrambled control lentivirus, in the DG of 6-8-week-old Nestin-CFPnuc mice housed under standard (resting) conditions. Ten days after injection, we found a higher percentage of Nestin-CFPnuc+ NPCs upon miR-135 inhibition, compared to mice injected with the scrambled control using flow cytometry (Sponge 1.6%, Figure 4.9A; Control 1.2%, Figure 4.9B). In another set of experiments (Figures 4.3-5), we administered BrdU (3 injections, every 2 hours) ten days after virus injection and killed the mice 24 hours after the first BrdU administration. We found in both control and miR-135a sponge-injected mice that >90 % of the BrdU-positive cells also expressed Nestin-CFPnuc (Figure 4.3B-D) and GFAP (Figure 4.3B), indicating that the majority of BrdU-positive cells in the SGZ of these mice were *bona fide* NPCs.

Figure 4.3: Next page - (A)) Relative expression levels of mature miR-135a in primary NPCs transduced *in vitro* with lentivirus transcribing the immature short-hairpin precursor of miR-135a (sh-miR- 135a, *i.e.*, gain of function), or a sponge for miR-135a (sponge miR-135a, *i.e.*, loss of function), or control viruses expressing scrambled RNAs. (**B**) Representative micrographs showing immunostaining for Nestin-CFPnuc (green), BrdU-positive cells (red), and GFAP-positive cells (white), and nuclear DNA with DAPI (blue) in the hippocampal SGZ of 6- to 8-week-old Nestin-CFPnuc mice, injected with lentiviruses (same used in A), kept 10 days under standard conditions and sub- jected to three injections of BrdU 24 hours before sacrifice. (**C**) Representative micrographs showing immunostaining for Nestin-CFPnuc (green), BrdU-positive cells (red) and nuclear DNA with DAPI (blue) in the hippocampal SGZ of 6- to 8-week-old Nestin-CFPnuc mice, injected with miR-135a sponge or scrambled- sponge lentivirus, kept 10 days under standard conditions, and subjected to three injections of BrdU 24 h before sacrifice. (**D**) Percentage of BrdU and Nestin-CFPnuc double-positive cells over total BrdU+ cells in the SGZ of mice injected with lentiviruses. Data are expressed as means ±SEM, n=7 mice per group. One-way ANOVA Bonferroni as post hoc. $^{**}p<0.01$, $^{***}p<0.001$. Scale bars, 50 µm (B) and 25 µm (C).

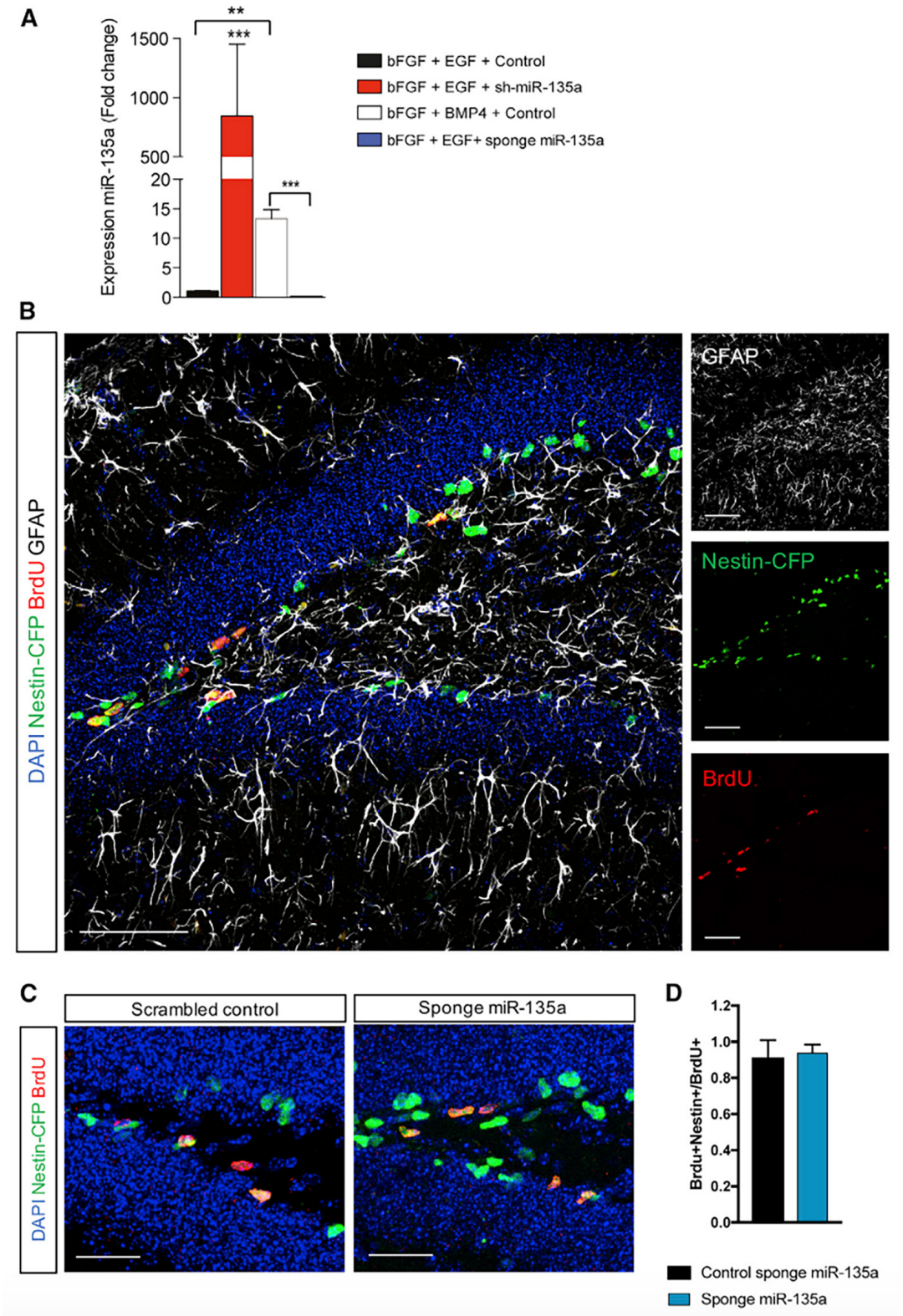


Figure 4.3: Validation of Lentiviruses to Overexpress/Downregulate miR-135a in NPCs *in vitro* and *in vivo*. Caption on previous page.

4.4 miR-135a Mediates Running- Induced Proliferation in the Hippocampal SGZ *in vivo*

Next, we assessed the effect of miR-135a manipulation on NPC proliferation *in vivo*. We injected viruses expressing sh-miR-135, sponge or scrambled controls into the DG of 8-week-old C57BL/6 mice and placed them in standard cages, or in cages equipped with a running wheel for ten days, followed by BrdU administration (Figure 4.4A).

In resting mice miR-135a inhibition led to a significant increase in the number of BrdU-positive cells in the SGZ, compared to controls (Figure 4.4B-C; $p<0.01$). In contrast, no significant differences in the number of BrdU-positive cells upon overexpression of miR-135a were observed (Figure 4.4B-C). The latter result could be explained by a saturation of the system due to the high expression levels of the endogenous miR-135a in NPCs (Figure 4.1). Importantly, we found that overexpression of miR-135a prevented the running-induced NPC proliferation in the SGZ (Figure 4.4B-C; $p<0.05$).

To corroborate these results, we analyzed the proportion of cells exiting the cell cycle upon manipulation of miR-135a in resting and running mice, by quantifying BrdU-positive cells that were negative for Ki67 in the SGZ (Figure 4.4B-D). Consistent with the antiproliferative function of miR-135a, we found a significant decrease in cell cycle exit upon injection with the miR-135a sponge in the DG of mice housed under standard conditions (Figure 4.4D; $p<0.05$). In contrast, overexpression of miR-135a significantly increased cell cycle exit in running mice (Figure 4D; $p<0.01$). In sum, these results indicate an antiproliferative function of miR-135a in NPCs and that its downregulation is necessary for the running-induced proliferation in the SGZ of adult mice.

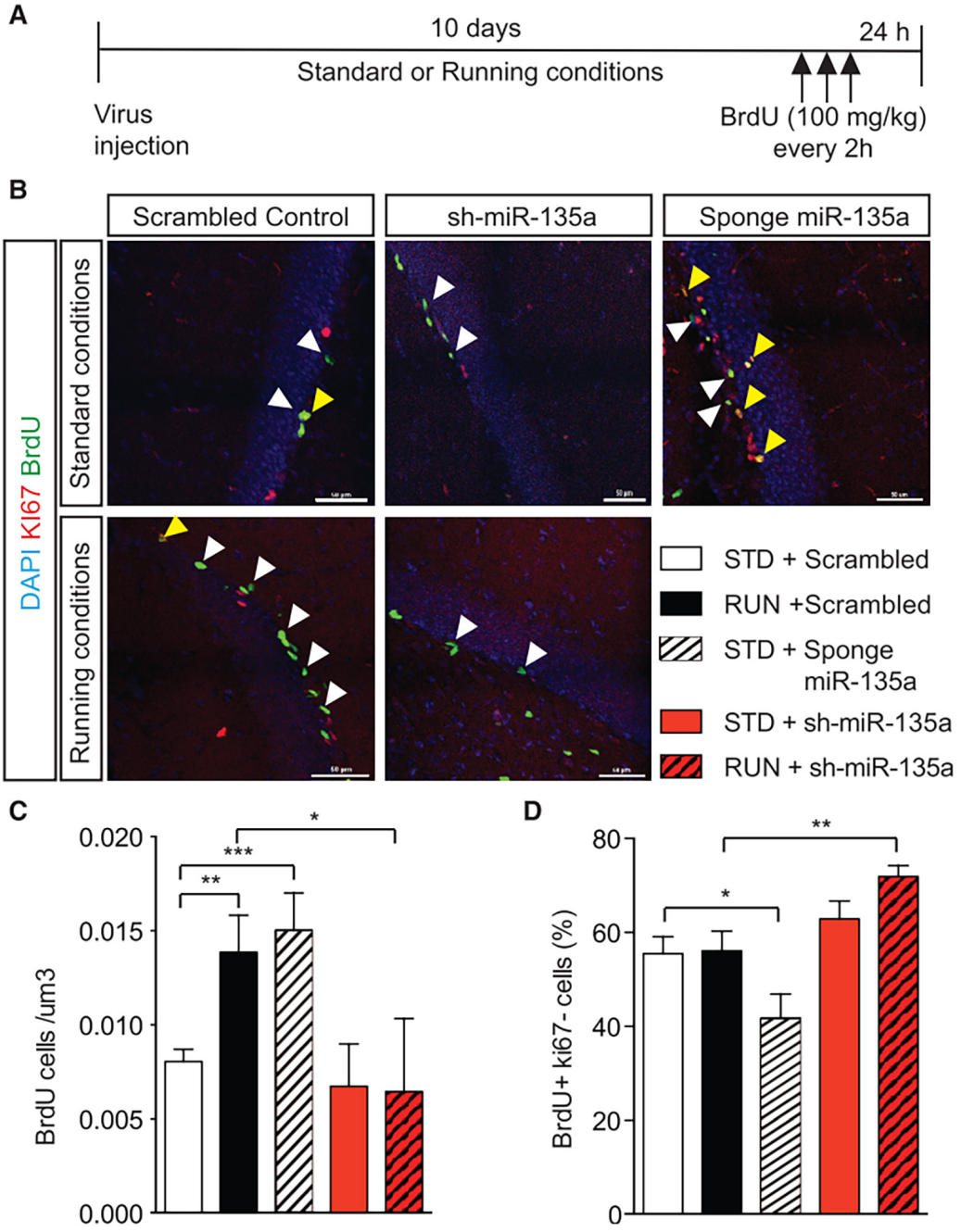


Figure 4.4: miR-135a Mediates Running- Induced Proliferation in the Hippocampal SGZ *in vivo*. (**A**) Schematic representation of the experiment. (**B**) Representative micrographs showing BrdU (green), Ki67 (red), or double-positive cells (yellow arrowheads) in the hippocampal SGZ of 6- to 8-week-old C57BL/6 mice, injected with scrambled, sponge miR-135a, or sh-miR-135a virus under standard or running conditions for 10 days and subjected to three injections of BrdU 24 h before sacrifice. White arrowheads, BrdU+ Ki67 cells; yellow arrowheads, BrdU+ Ki67+ cells. (**C**) Number of BrdU-positive cells per DG volume (mm^3). (**D**) Percentage of BrdU+Ki67 over total BrdU+ cells as a measure of cell-cycle exit. Data are expressed as means ±SEM, n=6 mice per group. One-way ANOVA Bonferroni as post hoc. $*p<0.05$, $**p<0.01$, $***p<0.001$. Scale bars, 50 µm.

4.5 Transient miR-135a inhibition stimulates hippocampal neurogenesis, but not astrogliogenesis *in vivo*

Next, we asked whether an increased proportion of proliferating NPCs, upon miR-135a inhibition, would also increase neurogenesis (Figure 4.5). Since constitutive inhibition of miR-135a prevents neuronal differentiation of NPC [192], we used synthetic "antagomiRs" to transiently inhibit miR-135a (anti-miR-135a), or scrambled control inhibitors in DG of resting mice and followed the fate of NPCs with BrdU (Figure 4.5A). As expected, injection of anti-miR-135a dramatically reduced endogenous miR-135a levels compared to control mice (Figure 4.5B; $p<0.0001$) and increased NPC proliferation, as indicated by higher number of BrdU+ cells in SGZ of mice (Figure 4.5C,D; $p<0.0001$).

Next, to evaluate the fate of NPCs, we quantified the proportion of cells co-expressing BrdU and the immature neuronal marker doublecortin (DCX), or the postmitotic neuronal marker NeuN in the DG three weeks after antagomiRs injection. Interestingly, we found that inhibition of miR-135a increased proportions of BrdU+DCX+(Figure 4.5E-G; $p<0.0001$) and BrdU+NeuN+ neurons, compared to control mice (Figure 4.5F-H; $p<0.05$). This result indicated that increased NPC proliferation, upon transient inhibition of miR-135a, leads to enhanced neurogenesis thus phenocopying running [159].

In contrast, miR-135a inhibition did not alter the proportion of BrdU+ cells expressing astrocyte markers such as Glial fibrillary acidic protein (GFAP) (Figure 4.10A-B), or glutamate transporter GLT-1 (*i.e.*, Solute Carrier Family 1 Member 2, SLC1A2) (Figure S4.3C-D) or the GLutamate ASpartate Transporter GLAST (*i.e.* Solute carrier family 1 glial high-affinity glutamate transporter member 3, SLC1A3) (Figure 4.10E-F). The latter result is consistent with our previous finding that adult hippocampal NPCs can undergo astrogliogenesis in absence of miRNAs [192].

Figure 4.5: Next page - (A)) Schematic representation of the experiment. (**B**) Relative expression levels of mature miR-135a in hippocampal DG of mice injected with control scrambled or anti-miR-135a 48 h after the injection. (**C** and **D**) (**C**) Representative micrographs showing BrdU (black or red) cells 6 days after injection of control (scrambled) or anti-miR-135a and (**D**) the number of BrdU+ NPCs per volume mm^3) in the hippocampal SGZ of 6-week-old C57BL/6 mice. (**E** and **F**) Percentage of BrdU+DCX+(**E**), BrdU+NeuN+(**F**) over total BrdU+ cells in the hippocampal SGZ of 6-week-old C57BL/6 mice 3 weeks after the injection with scrambled or anti-miR-135a antagomiRs. (**G** and **H**) Representative micrographs showing staining for BrdU (red), DCX (G, green) or NeuN (H, green); and nuclear DNA with DAPI (blue); arrowheads indicate double-positive cells. H, hilus, GCL, granular cell layer; SGZ, subgranular zone. Data are expressed as means ±SEM, n=7 mice per group. One-way ANOVA Bonferroni as post hoc. *$p<0.05$, ****$p<0.0001$. Scale bars, 50 mm and 25 mm (G, high magnification)

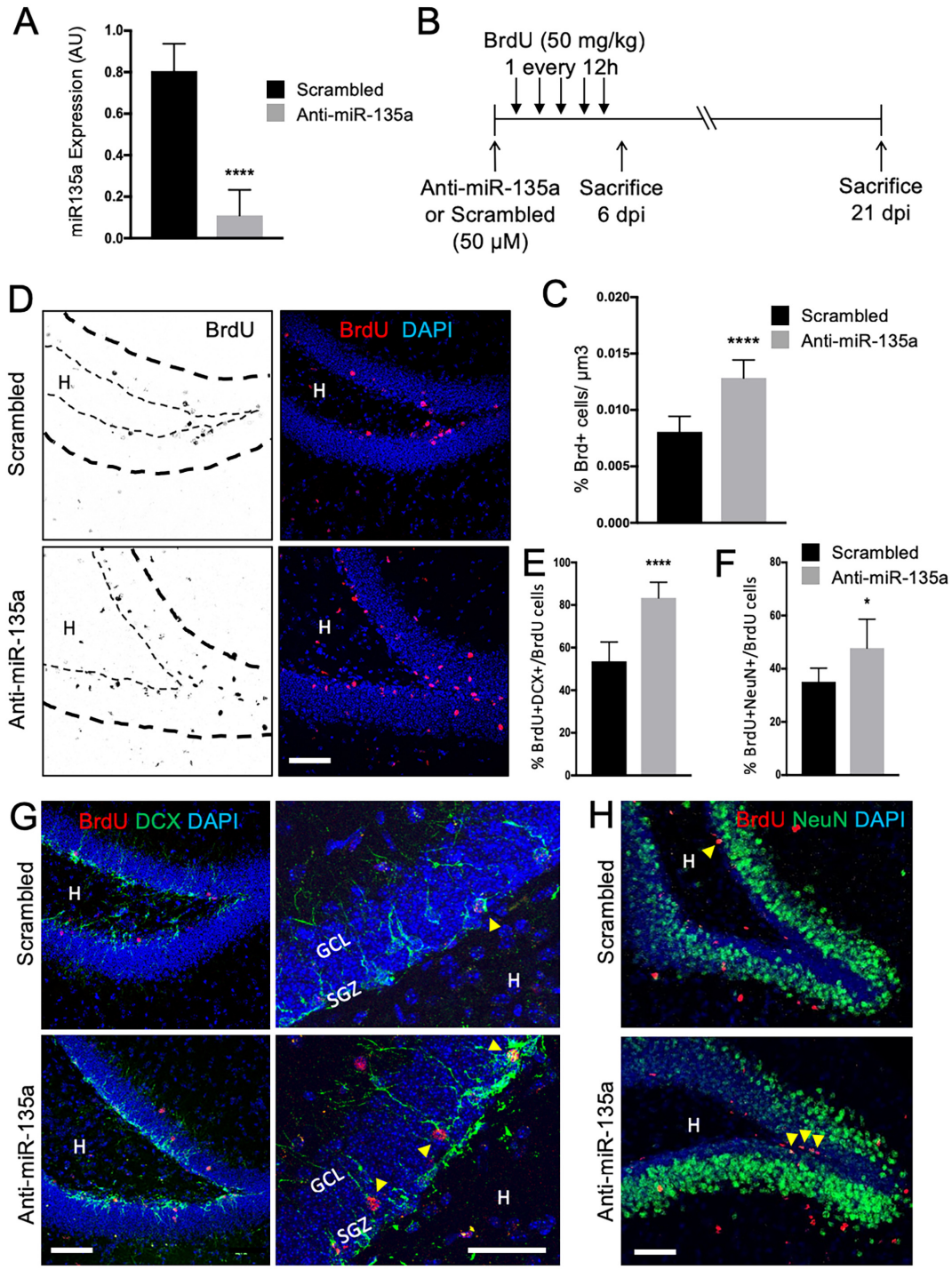


Figure 4.5: Transient miR-135a Inhibition Stimulates Hippocampal Neurogenesis *In Vivo*. Caption on previous page.

4.6 Inhibition of miR-135a reactivates proliferation in the hippocampal SGZ of aged mice and stimulates the re-entry of quiescent aNPCs into a proliferative state

The age-associated reduction in adult neurogenesis has been attributed both to exhaustion of the NPCs pools and/or to an increased state of quiescence of the remaining NPCs [18, 157, 193, 194]. Environmental stimuli have been shown to counteract the age-associated loss of adult neurogenesis in rodents, suggesting that a reversible cell cycle arrest of aged NPCs is possible at least to some extent. We hypothesized that miR-135a inhibition could restore proliferation in the SGZ in aged mice.

To verify this possibility, we injected either a lentivirus transcribing the sponge for miR-135a or the scrambled controls in the DG of eight-week-old (young) or 90-week-old (aged) C57BL/6 mice housed under standard conditions. Ten days later, we administered BrdU (3 injections, every 2 hours) and killed them 24 hours after the first injection (Figure 4.6). Remarkably, upon injection of the miR-135a sponge, both young and aged mice exhibited a similar significant increase in the number of BrdU-positive cells in the SGZ of the hippocampal DG compared to mice injected with the scrambled controls (Figure 4.6A). This result suggests that inhibition of miR-135a is sufficient to reactivate proliferation in the hippocampal SGZ of aged-mice.

To investigate whether inhibition of miR-135a stimulates cell cycle re-entry of quiescent NPCs, we infected primary cultures of hippocampal NPCs *in vitro* with lentivirus expressing the sh-miR-135a, the miR-135a sponge, or a scramble control RNA (Figure 4.6B-E). Quiescent NPCs have been proposed to accumulate genetic and epigenetic changes in histone and DNA over time [190]. To mimic this scenario, proliferating NPCs were first cultured in quiescence media for 72h, followed by 48h culture in new proliferative media (Figure 4.6B). Then, we measured the capacity of these cells to re-enter into a new proliferative state (Figure 4.6C-E), by quantifying the proportion of NPCs that were positive for BrdU (2h pulse, Figure 4.6C-D) or Ki67 (Figure 4.6C, E). Overexpression of miR-135a impaired NPCs proliferation re-entry, as shown by the lower percentage of BrdU- and Ki67- positive NPCs (Figure 4.6C-E), compared to cells infected with the scrambled control (Figure 4.6C-E; $p<0.001$, normalized to scrambled sh-control).

In contrast, inhibition of miR-135a upon infection with the miR-135a sponge, stimulated re-entry of NPCs in proliferation, as shown by higher percentage of BrdU- and KI67- positive NPCs, compared to the scrambled control (Fig-

ure 4.6C-E; $p<0.05$, normalized to scrambled sponge control). Consistently, cell cycle analysis using propidium iodide staining and FACS revealed that inhibition of miR-135a significantly increased the proportion of cells in S phase (10.24 % vs. 20.63 % $p<0.05$) and G2/M phases (9.63 % vs. 12.61 %; Figure 4.11A-B; $p<0.05$), at the expense of the G1/G0 phase (80.12 % vs. 66.55 %; Figure 4.11A-B; $p<0.01$), as compared to NPCs infected with the scrambled control.

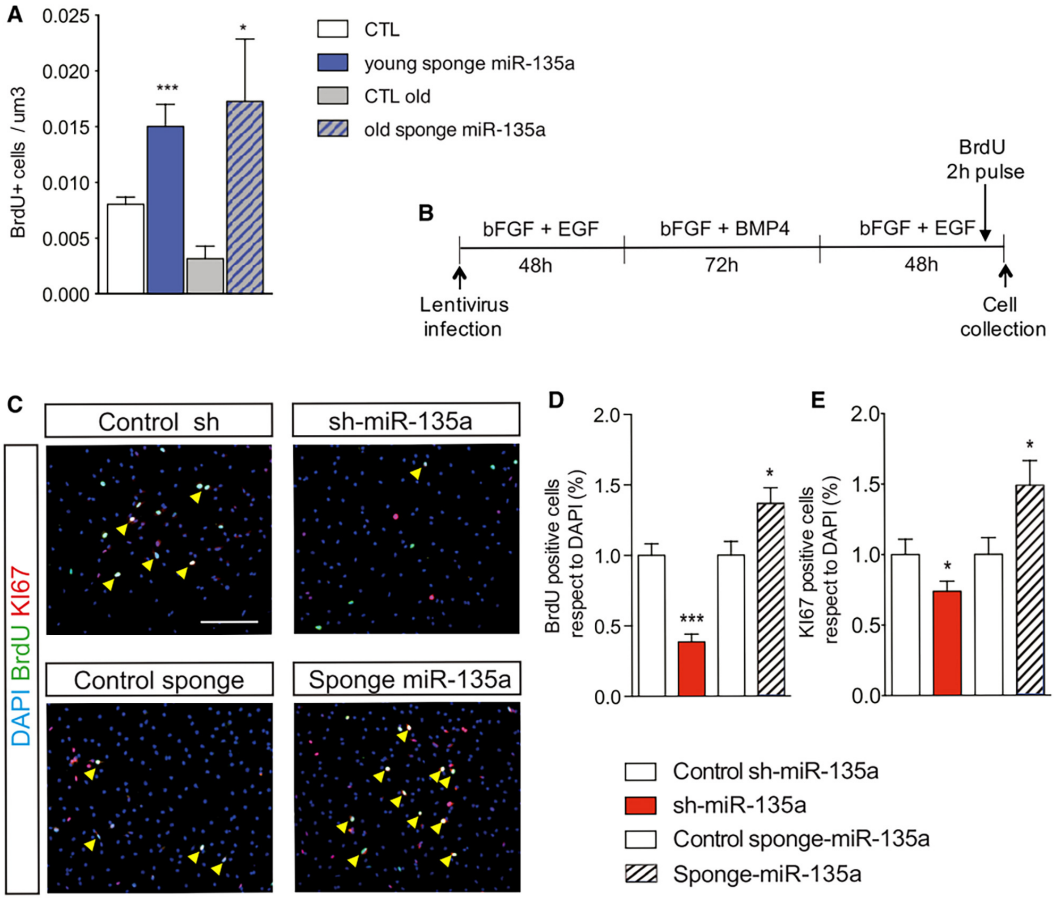


Figure 4.6: Inhibition of miR-135a Re-activates Proliferation in the Hippocampal SGZ of Aged Mice and Stimulates Cell-Cycle Re-entry of Quiescent NPCs. (**A**) Number of BrdU+ NPCs per DG volume (mm^3) in the hippocampal SGZ of 8-week-old (young) and 90-week-old (aged) C57BL/6 mice, injected with lentiviral sponge for miR-135a (loss of function), housed for 10 days under standard conditions, and subjected to BrdU administration (three injections every 2 h) 24 h before sacrifice. (**B**) Schematic representation of the in vitro experiment. Primary hippocampal NPCs were allowed to re-enter the cell cycle after 72 h in quiescence medium and fixed 2 h after BrdU administration. (**C**) Representative micrographs showing BrdU (green) and Ki67 (red) double-positive cells (yellow, arrowheads) of hippocampal NPCs infected with control sh-scrambled RNA, sh-miR-135a (gain of function), control sponge, or sponge miR-135a lentiviruses. (**D**) Percentage of BrdU+ cells relative to total cells (DAPI) normalized to controls. (**E**) Percentage of Ki67-positive cells relative to total cells (DAPI) normalized to controls. Data are expressed as means ±SEM, n=3 independent experiments containing three replicates. One-way ANOVA Bonferroni as post hoc. $^*p<0.05$, $^{***}p<0.001$. Scale bars, 50 µm.

Moreover, inhibition of miR-135a in NPCs led to a significant increase in the expression of transcripts encoding several markers of proliferation such as Ki67 and Mcm2, as well as candidate genes involved in cell cycle regulation such as cyclins A and cyclin E (Figure 4.11C).

Together, these results indicate that inhibition of miR-135a is sufficient to re-activate proliferation in the SGZ of aged mice, and that this might occur by stimulating quiescent NPCs re-entry into the proliferative state.

4.7 Phosphatidylinositol signaling acts downstream of miR-135a

To dissect proteins and pathways modulated by miR-135a in adult hippocampal NPCs, we first infected them with lentiviruses transcribing either the immature precursor or miR-135a (sh-miR-135), or the sponge for miR-135a and corresponding scrambled RNA controls (sh-control; sponge, control) *in vitro*, we then performed shotgun label-free proteomics analysis of extracts from these cultures. Upon overexpression of miR-135a, we found 431 up-regulated proteins (threshold >1.5-fold, Table S1) and 101 down-regulated proteins (threshold <0.5-fold; Figure 4.7A and Table S1, available in the online version of the publication); while upon inhibition of miR-135a we found 326 proteins up-regulated (>1.5-fold, Figure 4.7A and Table S2) and 109 down-regulated (<0.5-fold; Table S2).

To understand which mechanisms are affected by miR-135a in NPCs, we performed in silico GO analysis using DAVID bioinformatics resources [195,196] on all the proteins that were differentially expressed upon miR-135a manipulation (Tables S1 and S2). Top GO functions were: protein transport (GO: 001503 p-value sh-miR-135a 5.59E-06; p-value miR-135 sponge 3,29E-04); vesicle-mediated transport (GO:0016192; p-value sh-miR-135 5.34E-05; p-value miR-135 sponge 2.10E-03); transport (GO:0006810; p-value sh-miR-135 9.65E-03; p-value miR-135 sponge 5.10E-03) and nervous system development (GO:0007399; p-value sh-miR-135 9.79E-03; p-value miR-135 sponge 2.81E-02).

MiRNAs are mostly post-transcriptional repressors, hence, to identify the potential miR-135a targets in NPCs we focused on the proteins that were down-regulated upon overexpression of miR-135a and compared with those up-regulated upon its inhibition (Figure 4.7A-B). We found 17 proteins that were consistently affected by miR-135a levels in both data sets (Figure 4.7A-B). GO and KEGG pathway analysis of these proteins revealed that they are mostly involved in Intrinsic apoptotic signaling pathway in response to Endoplasmic reticulum stress (GO:0070059, p-value 0.0021, Figure 4.7C) and phosphatidylinositol signaling system (mmu:04070, p-value 0.0249, Figure 4.7C). Proteins predicted to be involved in these processes were inositol 1,4,5-trisphosphate (IP3) re-

A

B

ID protein (UniProtKB)	ID ENSEMBL	FULL NAME	Predicted Target?
ITPR1	ITPR1	Inositol 1,4,5-Trisphosphate Receptor Type 1	YES
RUFY3	RUFY3	RUN And FYVE Domain Containing 3	YES
MRCKB	CDC42BPB	CDC42 Binding Protein Kinase Beta	YES
ASTN1	ASTN1	Astrotactin 1	YES
F6V2U0	Inpp4a-213	Inositol Polyphosphate-4-Phosphatase Type I A	YES
CREL2	CRELD2	Cysteine-rich with EGF-like domain protein 2	NO TARGET
G3UWV4	BRSK2	BR Serine/threonine-protein kinase BRSK2	YES
EFR3B	EFR3B	EFR3 Homolog B	NO TARGET
EPDR1	EPDR1	Ependymin Related 1	NO TARGET
Q91V77	S100A1	S100 Calcium Binding Protein A1	NO TARGET
NOP9	NOP9	NOP9 Nucleolar Protein (Pumilio Domain-Containing Protein C14orf21)	YES
D3YTR6	SPNS1	Spinster homolog 1 (Putative sphingolipid transporter 1)	YES
TMED1	TMED1	Transmembrane P24 Trafficking Protein 1	NO TARGET
DYN1	DNM1	Dynamin 1	YES
CCD51	CCD51	Coiled-Coil Domain Containing 51	YES
Q9D1F0	RTL8B	CAAX box 1 homolog A	NO TARGET
HPF1	HPF1	Histone PARylation Factor 1	Yes

C

GO Biological process TERM: 0070059 ~ intrinsic apoptotic signaling pathway response to E.R. stress	P value: 0,02169	PROTEINS INVOLVED ON THE BIOLOGICAL PROCESS: **Brsk2 ; Itpr1**
mmu04070:Phosphatidylinositol signaling system	P value: 0,02497	PROTEINS INVOLVED ON THE KEGG PATHWAY: **Itpr1; Inpp4a**

D

Alignment miR-135a/b-5p: mmu (Mouse); Hsa (Human)	
mmu-miR-135b-5p	5'-UAUGGCUUUUCAUUCCUAUGUGA-3'
hsa-miR-135b-5p	5'-UAUGGCUUUUCAUUCCUAUGUGA-3'
mmu-miR-135a-5p	5'-UAUGGCUUUUUAUUCCUAUGUGA-3'
hsa-miR-135a-5p	5'-UAUGGCUUUUUAUUCCUAUGUGA-3'

E

miRNA-id	Ensembl-id	Gene	Start	End	Binding length	Longest pairings	Location
mmu-miR-135a-5p	ENSMUST00000078200	Brsk2	3492	3507	15	14	3UTR
	ENSMUST00000075528		3857	3872	15	14	
	ENSMUST00000018971		3397	3412	15	14	
mmu-miR-135b-5p	ENSMUST00000019441	Nop9	3273	3292	19	13	
hsa-miR-135a-5p	ENST00000074304	INPP4A	4399	4418	19	8	3UTR
	ENST00000409016		4282	4301	19	8	
	ENST00000409851		4384	4403	19	8	
hsa-miR-135a-5p	ENST00000357086	ITPR1	8002	8032	15	13	CDS
hsa-miR-135b-5p	ENST00000357086		7995	8032	15	13	
hsa-miR-135a-5p	ENST00000302640		8101	8131	15	13	
hsa-miR-135b-5p	ENST00000302640		8094	8131	15	13	
hsa-miR-135a-5p	ENST00000354582		8146	8176	15	13	
hsa-miR-135b-5p	ENST00000354582		8139	8176	15	13	
hsa-miR-135b-5p	ENST00000417478	RUFY3	652	676	24	8	CDS
	ENST00000381006		1204	1228	24	8	CDS
	ENST00000226328		4015	4058	43	11	3UTR
	ENST00000226328		1204	1228	24	8	CDS
	ENST00000502653		596	620	24	8	CDS
hsa-miR-135a-5p	ENST00000361246	CDC42BPB	3509	3537	28	10	CDS
hsa-miR-135b-5p	ENST00000361833	ASTN1	6434	6457	23	10	3UTR
hsa-miR-135b-5p	ENST00000531197	BRSK2	3223	3242	19	13	3UTR
	ENST00000528841		3646	3665	19	13	
	ENST00000308219		3176	3195	19	13	
	ENST00000528710		3210	3229	19	13	
	ENST00000382179		3603	3622	19	13	
hsa-miR-135b-5p	ENST00000267425	NOP9	4611	4630	19	12	3UTR
	ENST00000396802		4445	4464	19	12	
hsa-miR-135b-5p	ENST00000341179	DNM1	806	833	27	7	CDS
	ENST00000372923		806	833	27	7	
	ENST00000475805		806	833	27	7	
	ENST00000393594		806	833	27	7	
	ENST00000486160		806	833	27	7	
	ENST00000627543		806	833	27	7	
	ENST00000628346		806	833	27	7	
hsa-miR-135a-5p	ENST00000566059	SPNS1	827	856	18	9	CDS
hsa-miR-135b-5p	ENST00000566059		827	856	18	9	
hsa-miR-135b-5p	ENST00000566059		625	645	20	9	
hsa-miR-135a-5p	ENST00000565975		807	836	18	9	
hsa-miR-135b-5p	ENST00000565975		788	836	18	9	
hsa-miR-135b-5p	ENST00000565975		620	640	20	9	
hsa-miR-135a-5p	ENST00000311008		1041	1070	18	9	
hsa-miR-135b-5p	ENST00000311008		1022	1070	18	9	
hsa-miR-135b-5p	ENST00000311008		854	874	20	9	
hsa-miR-135a-5p	ENST00000334536		1041	1070	18	9	
hsa-miR-135a-5p	ENST00000352260		975	1004	18	9	
hsa-miR-135b-5p	ENST00000352260		956	1004	18	9	
hsa-miR-135b-5p	ENST00000352260		788	808	20	9	
hsa-miR-135a-5p	ENST00000323081		954	983	18	9	
hsa-miR-135b-5p	ENST00000323081		935	983	18	9	
hsa-miR-135b-5p	ENST00000323081		767	787	20	9	
hsa-miR-135b-5p	ENST00000393381	HPF1	234	271	37	11	CDS
hsa-miR-135b-5p	ENST00000442740	CCDC51	332	386	24	12	5UTR
hsa-miR-135b-5p	ENST00000395694		933	955	22	9	CDS

Figure 4.7: Phosphatidylinositol Signaling Proteins Are Modulated by miR-135a in NPCs. (**A** and **B**) Venn diagram indicating the number of downregulated proteins (purple, <0.5-fold) upon overexpression of miR-135a (sh-miR-135a), or upregulated proteins (yellow, >1.5-fold) upon inhibition of miR-135a (Sponge-miR-135a), and the 17 differently expressed proteins (table in B) found in both datasets, in cultured primary hippocampal NPCs. (**C**) In silico GO analysis and KEGG pathway analysis. (**D**) Alignment of mouse and human miR-135a-5p and miR-135b-5p. (**E**) Predicted targets of miR-135 according to MiRWalk. Position and length of predicted target sites of miR-135a-5p and miR-135b-5p are shown for each transcript (Ensembl ID).

ceptor 1(ITPR1) and BRSK2, or inositol polyphosphate-4-phosphatase, type I (INPP4A), respectively. Interestingly, the transcript encoding for Human INPP4A (Transcript ENST00000409016; Gene IDENSG00000040933) is an experimentally validated target of miR-135a-5p (Target site position: chr2:98590068-98590095, DIANA-Tarbase v8.0) [197] in human brain cortex [198]. This gene encodes an Mg^{++} independent enzyme that hydrolyzes the 4-position phosphate from the inositol ring of phosphatidylinositol 3,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 3,4-bisphosphate.

To identify additional potential miR-135a targets amongst the 17 candidate proteins, we used prediction software miR-Walk 3.0, [199]. Since both miR-135a-5p and miR-135b-5p share identical seed regions and are conserved in human and mouse (Figure 4.7D), we searched targets in both human and mouse databases (Figure 4.7E). We found that 11 of 17 affected proteins upon miR-135a manipulation are predicted targets of this miRNA (Figure 4.7E). Finally, we repeated the same analysis workflow for the proteins upregulated (>1.5-fold, Table S1) upon miR-135a overexpression and downregulated upon miR-135a inhibition (<0.5-fold, Table S2). We found 23 proteins that were consistently affected by miR-135a levels in both data sets, but in contrast only 4 of them (17 %), were predicted targets of (either human or mouse) miR-135a-5p or miR-135b-5-p (not shown), suggesting these proteins are indirectly modulated by miR-135.

4.8 Discussion

In this study, we identify miR-135a as the first noncoding RNA essential modulator of the brain response to physical exercise. We report that overexpression of miR-135a in the DG prevents running-induced NPC proliferation. On the other hand, miR-135a inhibition stimulates NPC proliferation leading to increased neurogenesis, but not astrogliogenesis, in DG of resting mice. Remarkably, miR-135a inhibition reactivates NPC proliferation in DG of aged mice, likely by stimulating quiescent NPC pools to re-enter the cell cycle.

Several studies reported altered hippocampal miRNA expression in response to physical exercise [162, 163, 165], orpathological conditions [96]. To our knowledge, this is the first study reporting a functional role of one miRNA underlying the exercise-mediated increase in adult neurogenesis. Functions of the two members of the miR-135 family are poorly described in the mammalian CNS. In postmitotic neurons, miR-135 regulates axon growth/regeneration and mediates long-term depression [200, 201]. miR-135a expression is high in the amygdala of stressed mice [202], and in the mouse raphenucleus (functionally connected to the hippocampus) it is a key regulator of serotoninergic networks and antidepressants action [203]. However, since miR-135 association with

depression- and anxiety-related phenotypes in patients is very variable [204], its role in the pathological mechanism of these diseases remains unclear. In the adult mouse hippocampus, miR-135 is rapidly upregulated after prolonged kainic acid-induced seizures [40].

Physical exercise is a potent trigger of adult hippocampal neurogenesis in both young and aged mice, but cellular and molecular mechanisms underlying this phenomenon remain controversial. Cellular mechanisms include recruitment of quiescent neural stem cells, acceleration of the cell cycle of NPCs, increased number of cell divisions, and reduction of cell death [205]. At the molecular level, physical exercise has been shown to increase levels of growth factors BDNF, IGF, FGF-2, and VEGF, leading to the activation of MAPK/ERK and PI3K-Akt signaling pathways [206]. We report that INPP4A, a key enzyme for phosphatidylinositol metabolism and known target of miR-135 in the cortex [198], is one of the top proteins modulated by miR-135 in NPCs. ITPR1, another differentially expressed protein identified in our analysis, is also a key player in IP3 signaling.

Hence, phosphatidylinositol signaling could represent a prominent constraint to NPC proliferative potential. This hypothesis is consistent with previous studies indicating that the PI3K-Akt signaling pathway is activated by exercise in rodents [207, 208]. However, while these studies concluded that the PI3K-Akt pathway primarily mediates the effect of exercise on the survival of newly generated DG neurons and the associated increase in synaptic plasticity, our results suggest that miR-135/phosphatidylinositol signaling could mediate exercise-induced proliferation of NPCs.

Together, this evidence opens the possibility that the miR-135-IP3-axis might represent a novel target of therapeutic intervention to stimulate adult neurogenesis and therapeutic exploitation of miR-135 might offer intriguing perspectives to delay or prevent pathological brain aging. One unanswered question arising from our study is how running decreases miR-135 levels in adult NPCs. miR-135 is a tumor suppressor [209, 210], which is downregulated in several cancers [211–215].

4.9 Supplementary informations

A

Target name	EXP1 Ct values		EXP2 Ct values		EXP3 Ct values	
	STD	RUN	STD	RUN	STD	RUN
miR-135a	24,142	32,448	20,975	21,225	19,784	19,696
miR-375	30,814	35,344	27,745	28,186	26,388	25,953
miR-203	32,996	34,865	28,829	29,491	27,756	27,136
miR-190	23,909	24,855	21,233	21,528	17,267	21,292
miR-381	29,31	29,699	25,307	25,398	25,04	24,774
miR-350	28,429	28,709	24,308	24,452	24,542	23,719
miR-340-3p	26,755	26,95	21,624	21,862	21,343	20,338
miR-31	29,469	29,351	25,332	26,311	23,661	23,305
Normalizators						
U87-001712	22,620	22,193	20,092	19,034	20,203	18,246
Y1-001727	22,126	21,635	19,849	20,545	18,988	17,653
snoRNA135-001230	22,995	22,45	13,905	14,005	14,403	12,984
snoRNA202-001232	16,457	16,336	20,2	19,495	19,845	18,458

B

Target name	STD1	STD2	STD3	RUN1	RUN2	RUN3	t-test (paired)
miR-135a	0,348	0,542	1,337	0,001	0,219	0,366	0,123
miR-190	0,003	0,005	0,014	0,000	0,002	0,005	0,113
miR-203	0,057	0,346	0,454	0,037	0,141	0,235	0,147
miR-375	0,409	0,453	7,653	0,158	0,178	0,121	0,383
miR-381	0,001	0,002	0,005	0,000	0,001	0,002	0,140
miR-350	0,010	0,027	0,035	0,006	0,012	0,011	0,130
miR-340-3p	0,018	0,054	0,049	0,011	0,023	0,023	0,100
miR-31	0,009	0,026	0,091	0,007	0,006	0,030	0,257

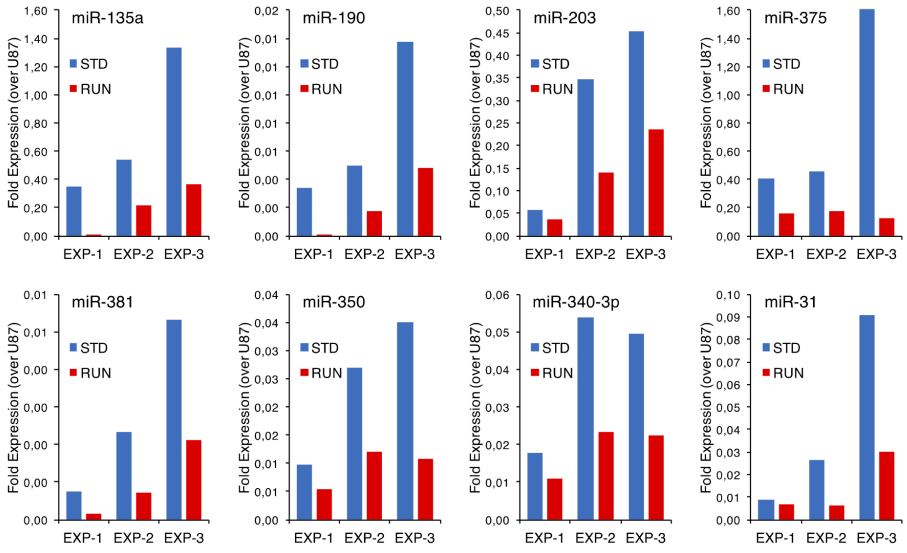


Figure 4.8: Fold change expression of miRNAs in Nestin-CFPnuc positive NPCs sorted from adult hippocampus of mice in standard (STD) or running (RUN) conditions for 10 days. (**A**) Expression levels of miRNAs and normalizators (Ct values) by TLDA from each independent experiment (EXP; n=3 independent biological replicas each of them containing a pool of Nestin-CFPnuc positive cells isolated from 8 mice, per condition). (**B**) Normalized values from each independent experiment and plots.

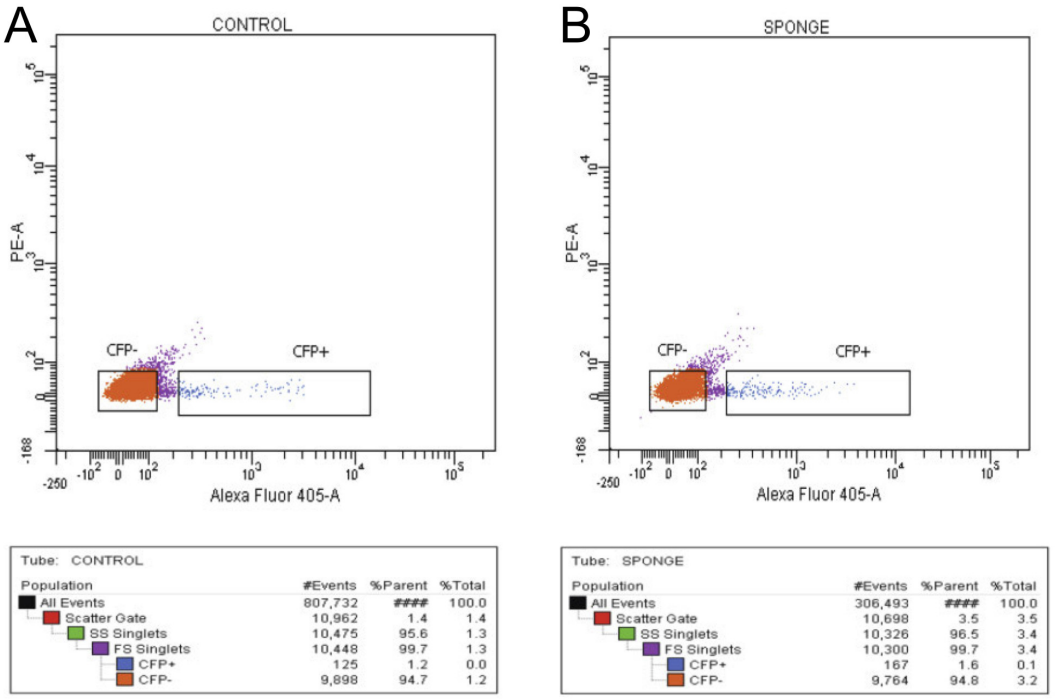


Figure 4.9: Inhibition of miR-135a increases the number of Nestin-CFPnuc positive NPCs *in vivo*. (**A**-**B**) Fluorescent sorting of CFPnuc positive cells from 6-week-old Nestin-CFPnuc mice after 10 days upon stereotaxic injections of lentivirues encoding control (**A**), or miR-135a (**B**) sponge in the hippocampal DG of adult mice. n=10 mice per group, pooled before sorting.

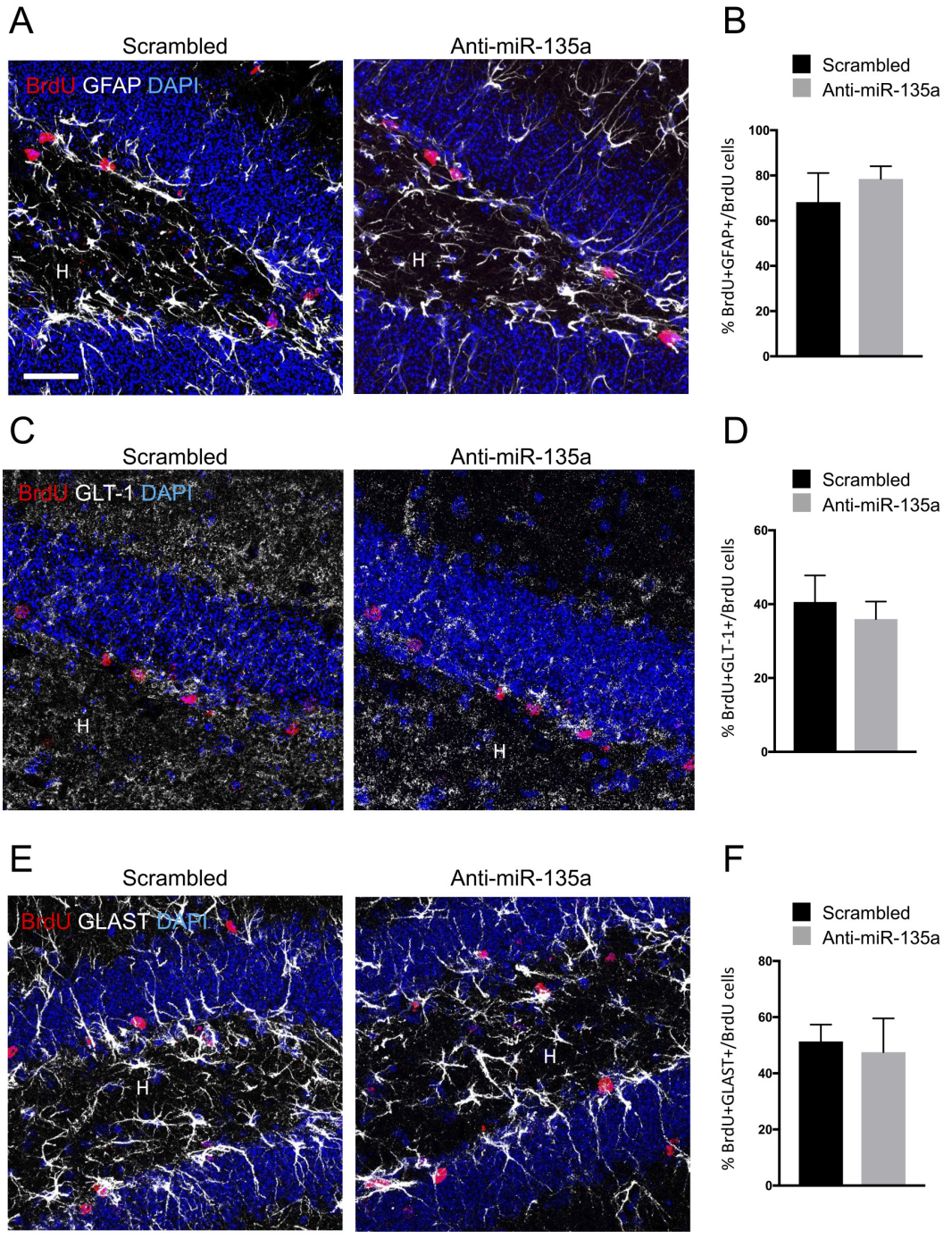
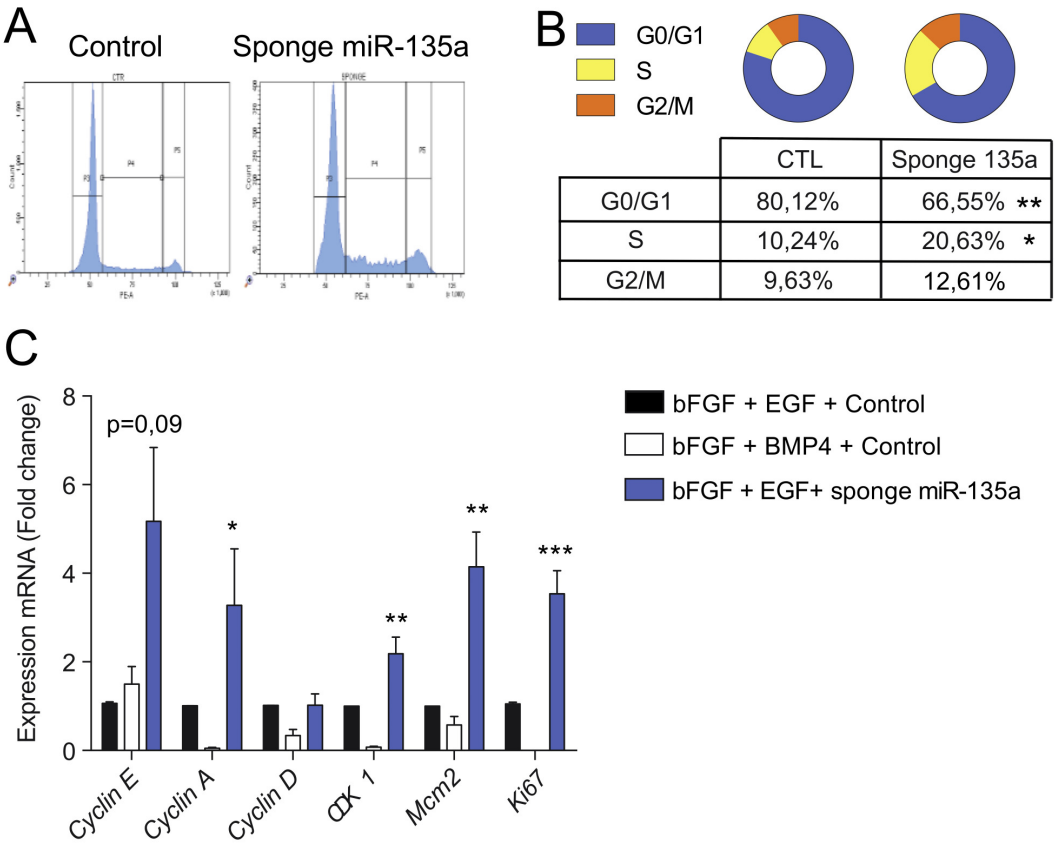


Figure 4.10: Transient inhibition of miR-135a does not alter the astrogliogenesis in hippocampal SGZ. (**A-F**) Representative micrographs showing BrdU (red), GFAP (A), GLT-1 (C) or GLAST (E) (white) cells in hippocampal SGZ of 6-weeks-old C57BL/6 mice, injected with control scrambled or anti-miR-135a, subject to BrdU administration (2 injections every day per 5 days) and sacrificed 2 weeks after the last BrdU injection. (B) Quantification of the proportion BrdU+GFAP+ (B), BrdU+GLT-1+ (D) or BrdU+GLAST+ over total BrdU+ cells. H, hilus. Data are expressed as mean pmSEM, n=7 mice per group. One-way ANOVA Bonferroni as post hoc. *p<0.05, ****p<0.0001. Scale bars, 25 µm.



	CTL	Sponge 135a
G0/G1	80,12%	66,55% **
S	10,24%	20,63% *
G2/M	9,63%	12,61%

Figure 4.11: Downregulation of miR-135a regulates proliferation and cell cycle -dependent genes in NPCs *in vitro*. (**A**) Representative cell cycle analysis of propidium iodide staining by flow cytometry. (**B**) Percentage of NPCs in G0/G1, S and G2/M phases upon infection with viral-encoded control or sponge for miR-135a *in vitro*. (**C**) Quantification of relative mRNA expression of cell cycle-dependent genes normalized to Actin in NPCs cultured in proliferative (bFGF+EGF), or quiescence (bFGF+BMP4) media upon transduction with viral-encoded control, and in proliferative media upon transduction with viral-encoded miR-135a sponge. Data are expressed as mean ±SEM, n=3 independent experiments containing three replicates. One-way ANOVA Bonferroni as post hoc. *p<0.05, **p<0.01, ***p<0.001.

5 Conclusions and perspectives

Lifelong maintenance of neurogenesis has implication for memory, ageing and age-related diseases. Interestingly, newborn neurons are still generated in old human brains, and the aged mouse brain retains some ability to regulate neurogenesis in response to macroenviromental changes, such as exercise [216]. Mechanisms proposed to explain the age-related drop of adult neurogenesis include deforestation of aNPC through their conversion into reactive astrocytes [18], a phenomenon accelerated by epilepsy [34] and neurodegeneration [217, 218].

One hallmark of brain aging and age associated neurodegenerative disorders is gliosis: reactivity in astrocytes typically increases their inflammatory phenotype and cause loss of their neuro-supportive functions, thus rendering neurons vulnerable to hypo-metabolic states, excitotoxicity and oxidative stress [219]. Aging is associated with increased levels of Reactive oxygen species (ROS), oxidized products and reactive nitrogen species (RNS) in different tissues. Indeed, the oxidative stress theory of aging is based on the hypothesis that age-associated functional losses are due to the accumulation of reactive oxygen and nitrogen species (RONS) -induced damages. RONS are important regulators of cellular metabolism, gene expression, and other molecular responses, playing key roles in the control of various physiological processes. Oxidative stress is involved in several age-related conditions (*i.e.*, cardiovascular diseases, chronic obstructive pulmonary disease, chronic kidney disease, neurodegenerative diseases, and cancer), including sarcopenia and frailty [220, 221]. Therefore, it becomes clear that understanding the regulation of RONS in aging is crucial.

Within this context, our results indicate that the Piwi pathway prevents glial reactivity in the postnatal hippocampus. Glial reactivity causes loss of their neuro-supportive functions, thus rendering neurons vulnerable to metabolic and oxidative stress [219]. In addition, dysregulated expression of the Piwi pathway has been associated with various neuronal disorders, such as Alzheimer's disease and Rett Syndrome; moreover, piRNAs could represent potential therapeutic targets and appropriate diagnostic markers of these diseases [139]. Interestingly, a piRNA-miRNA signature in circulating exosomes has been recently proposed as a hallmark of human neurodegeneration: this is an important finding, useful to detect Alzheimer's disease on the basis of cerebrospinal fluid samples and it may also help to predict conversion of mild–cognitive impaired patients [222].

Another important aspect of aging is its direct link to DNA damage, which may be caused by the transposition of TEs. TEs are powerful drivers of genome evolutionary dynamics but are principally deleterious to the host organism by

compromising the integrity and function of the genome. The Piwi pathway can be considered also as a widespread strategy used by most animals to effectively suppress transposition [223]. In the absence of active Piwi pathway, aging somatic cells tend to increasingly lose heterochromatin, which normally maintains TEs under transcriptional repression [224, 225]. Even if TEs are not the major targets of Piwi pathway in aNPCs, our data indicate an increased proportion of TEs-derived piRNAs in differentiating neuroblasts, compared with proliferating cells. This evidence is in line with previous data reporting a high expression and activity of TEs during neurogenesis [141,150]. Additional studies will be needed to caratterize the regulation of TEs activity and DNA damage in brain aging, to better understand the possible role of Piwi pathway in protecting somatic stem cells from TE-mediated mutagenesis.

Beside piRNAs and among various epigenetic events, also miRNAs turned out to be important players in controlling ROS and aging: miRNAs can generate rapid and reversible responses and, therefore, are ideal players for mediating an adaptive response against stress through their capacity to fine-tune gene expression [226]. About 70 miRNAs are upregulated in murine brain aging, starting from 18 months of age [227]. Consistent with this evidence, our study showed that miR-135a inhibition reactivates aNPC proliferation in DG of aged mice, likely by stimulating quiescent aNPC pools to re-enter the cell cycle.

A possible mechanisms to explain the rescue of proliferation in the hippocampal stem cell niche of aged mice upon miR-135 downregulation, is provided from the recent identification of a Wnt/miR-135a auto-regulatory loop in brain development, which could modulate differentiation of forebrain [228] and dopaminergic neurons [229, 230]. Moreover, Wnt/TGFβ/BMP pathways are known to influence long- term maintenance of NPC pools in the adult hippocampus, age-associated cognitive decline, and brain dysfunctions [231,232]. Furthermore, we recently found that miR-135a is one of the 11 miRNAs required and sufficient to sustain the neurogenic lineage fate of NPCs [97]. Hence, it appears that miR-135 is required to regulate multiple aspects of adult hippocampal neurogenesis, suggesting that further studies on miR-135 in the brain response to physiological and pathological conditions are warranted.

As mentioned before, pathological conditions such as epilepsy and neurodegeneration worsen the drop of adult neurogenesis in brain aging. Adult hippocampal neurogenesis is strongly affected by epilepsy, in terms of enhanced network excitability, increased short-term and reduced long-term proliferation rate of NSCs, due to their direct conversion into astrocytes. In addition, newborn neurons also display morphological and functional alterations (reviewed in [10]). Several studies reported an alteration of global miRNA expression in epileptic brains from a variety of animal models and humans. Most of these miRNAs are

already known to regulate hippocampal neurogenesis and are involved in cellular processes relevant to the disease, such as cell proliferation and migration, neuroinflammation, and neuronal apoptosis (reviewed in [233]).

In this view, many researchers are struggling to use miRNA mimic or inhibitor sequences as possible therapeutic molecules in epilepsy, although several limits are emerging (*i.e.*, targeted delivery, multi-targeting effect). Latterly, a recent study reported upregulated level of miR-135a in neurons within the epileptic brain. Targeting miR-135a *in vivo* with antagomirs after onset of spontaneous recurrent seizures, can reduce their insurgence in chronic epilepsy [234]. These data support the exciting possibility that miRNAs can be targeted to treat epilepsy.

Outstandingly, we found dysregulated expression of the Piwi pathway in hippocampal aNPCs after treatment with kainic acid, that induces neuronal hyperexcitation similar to epilepsy. Our data correlates aberrant gliosis and depletion of the Piwi pathway in normal and impaired hippocampal neurogenesis. This evidence opens new intriguing prospective in the use of Piwi proteins and piRNAs as modulators of aNPCs homeostasis.

In sum, we uncovered the role of noncoding RNAs (piRNAs and miR-135a) in the regulation of gliosis and exercise-mediated proliferation within adult neurogenesis. Several studies have shown other mechanisms able to promote neurogenesis and improve rodent cognitive function in the aged brain, such as restoring trophic factors [3], decreasing humoral aging factors [235] or injecting plasma from younger animals [236].

These findings imply that aged stem cells may still have the potential to aid in recovery from injury and disease but are restricted in how much they can grow and for how long [237]. Indeed, our results contribute to the identification of the mechanism involved in age- and pathology-related cognitive decline and could enhance the application of adult neurogenesis for therapeutic use. Further studies on the brain-Piwi pathway and the miR-135a neuronal functions in health and disease are warranted.

Part III

Procedures and References

6 Experimental Procedures

6.1 Animals and running

Male and female C57BL/6, Td-Tomato$^{flox/wt}$ knock-in reporter mice (Jackson Laboratory stock number 007908) [238] and Nestin-GFP mice [176], were housed under standard laboratory conditions at Istituto Italiano di Tecnologia (IIT), the Center for Regenerative Therapies Dresden (CRTD), the Medizinische-Theoretisches Zentrum (MTZ) Dresden or at the Swammerdam Institute for Life Sciences, University of Amsterdam. All experiments and procedures were approved by the Italian, German and Dutch authorities (permit nos. 056/2013, 214/2015-PR, 24-9168.11-1/2013-15, and AVD1110020184925), and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the European Community Council Directives, in accordance to European Union (EU) directive 2010/63/EU. Mice were maintained under a 12 hours light/dark cycle with food and water ad libitum.

Running: nine-week-old Nestin-CFPnuc mice or WT C57BL/6J were double housed under standard conditions or in cages equipped with a running wheel (TSE System, animal facility of CRTD; or ENV-044 [Med Associates], Animal facility of IIT) for 10 days before sacrifice. 24 hours before sacrifice, mice received one administration of 50 mg/kg BrdU (B9285, Sigma).

6.2 aNPCs preparation and culture

Hippocampal NPCs were prepared and expanded as described previously [97, 189,239]. Briefly, DG was isolated from 8-10 wild-type C57BL/6 mice at the age of 6-8 weeks. After dissection in Hanks Balanced Salt Solution (Hank's Balanced Salt Solution (HBSS), Gibco) medium, the tissue was enzymatically dissociated with papain (2.5 U/ml), dispase (2.5 U/ml) and DNaseI (250 U/ml) for 20 min at 37 °C. During incubation, the tissue was repeatedly triturated with a fire polished Pasteur pipette. The cell suspension was centrifuged at 130 g for 5 min and the pellet was re-suspended in buffer solution (1x HBSS, 30mM Glucose, 2mM HEPES pH 7.4, 26mM $NaHCO_3$) followed by a centrifugation at 130 g for 5 min. aNSCs were isolated using 22 % Percoll gradient solution.

After further centrifugation for 5 min at 130 g the cell pellet was re-suspended in 2 ml of culture medium containing Neurobasal (Invitrogen), Glutamax (Invitrogen), 1 % penicillin and streptomycin (Invitrogen), B27 without retinoic acid (Invitrogen), FGF (20 ng/ml; PeproTech) and EGF (20 ng/ml; PeproTech). The dissociated DG tissue was plated into PDL/Laminin (Sigma/Roche) coated

wells and incubated at 37 °C with 5 % CO_2. To further remove excess debris, the growth medium was exchanged 24 hours later. Every 2 days half of the growth medium was exchanged with fresh medium to replenish the growth factors. aNSCs were passaged once they reached 80 % confluence.

Proliferation medium: Neurobasal, Glutamax, 1 % penicillin and streptomycin, B27 without retinoic acid, supplemented with FGF (20 ng/ml) and EGF (20 ng/ml).

Retrovirus-mediated inducible neuronal differentiation: viral construct expressing Ascl1-ERT2 and infections conditions were previously described [171]. Neuronal differentiation was induced by growth factor withdrawal in the presence of 0.5 mM OHTAM (Sigma) for 2 days. The medium was changed every 2–3 days. Cells were fixed at 4, 7, 14 or 21 days after the exposition to OHTAM.

Neuronal/astrocytic differentiation: aNPCs were plated at 1,2*10E4 cells/cm^2 in culture medium supplemented with FGF (20 ng/ml) 24 hours. Then medium was exchanged with medium containing B27 with retinoic acid and FGF (5 ng/ml) for 24 hours and FGF (1 ng/ml) during the next four days. Cells were differentiated in culture for 7, 14 and 21 Days *in vitro* (DIV).

6.3 miRNA Manipulation

Scrambled miRNA mimics (50 nM; QIAGEN) or miRNA antagomiRs (150 nM; QIAGEN) were nucleofected (Amaxa) in proliferating or quiescent NPC cultures. Then, 48 h after nucleofection, 10 mM BrdU was added to the medium for 2 h, followed by fixation (4 % ParaFormAldehyde (PFA)) or RNA/protein extraction. MiRNA mimics (Qiagen): Syn-mmu-mir-135a-5p, MSY0000147; Syn-mmu-miR-203-3p, MSY0000236; or Syn-mmu-miR-190a-5p, MSY0000220. MiRNA antagomirs (Qiagen): Anti-mmu-miR-135a-5p, MIN0000147; Anti-mmu-miR-203-3p, MIN0000236; or Anti-mmu-miR-190a-5p, MIN0000220.

To constitutively overexpress or inhibit miR-135a-5p, NPCs were infected at Multiplicity Of Infection (MOI)=5 with lentiviruses and selected with 1 µg/ml puromycin (Sigma). Cell cycle distribution was monitored by propidium iodide staining of cells and fluorescence-activated cell sorting (FACS).

6.4 Protein extraction and Western blot

For total protein extraction, adult testis or hippocampus or cell pellets were homogenized in RIPA buffer. Testis and hippocampus were sonicated (10 short pulses) and left on ice for 15 min. Cell pellets were clarified by centrifugation at 20 000 g, and the protein concentration was determined using a Bradford Assay kit (Bio-Rad).

Antibody	Host	Company	Catalog	Dilution
MILI	Mouse	Hannon Lab		1:100
MIWI	Rabbit	Hannon Lab		1:200
ACTIN	Rabbit	Abcam	ab13970	1:1000
GADPH	Rabbit	Santa Cruz	sc-25778	1:1000
GFAP	Rabbit	Dako	Z-0334	1:1000

Table 6.1: List of antibodies used for WB

For blot analysis, equal amounts of protein (30 µg) were run on homemade 10 % polyacrylamide gels and transferred on nitrocellulose membranes (GE Healthcare). Membranes were probed with primary antibodies (listed in the table below) followed by HRP-conjugated secondary antibody anti-rabbit or mouse (Invitrogen, A16104, A16072; 1:2,000). LAS 4000 Mini Imaging System (GE Healthcare) was used to digitally acquire chemiluminescence signals, and the band intensities were quantified using Fiji.

6.5 Virus injection and tissue preparation

Virus injection: to label aNPCs in adult DG, 8 weeks-old mice Td-Tomato$^{flox/wt}$ were anesthetized with isoflurane and 1 µl of virus mix (Split-Cre N-Cre:C-Cre) per DG was stereotaxically injected at the following coordinates: −2.0 anterior/posterior, ±1.6 medial/lateral, and −1.9 to −2.1 dorsal/ventral relative to bregma (in millimeters) as previously described [169]. Mice were single-housed under standard conditions or in cages equipped with running wheels for 10 days. For piRNA project, animals were sacrificed 10 days or 1 month after BrdU injections and used for DG extraction and molecular analysis or histology. For miR-135 project, after virus injection, mice received three BrdU intraperitoneal injections per day (100 mg/kg, every 2 h) and sacrificed 24 h later. After injection of oligos, mice received two BrdU intraperitoneal injections per day (50 mg/kg) for 5 days and sacrificed 2 h (6 days post injection of oligos) or 2 weeks after the last BrdU injection (21 days post injection of oligos).

Virus used: mmu-miR-135a-5p sponge: MISSION® Lenti microRNA Inhibitor Mouse (MLTUD0048, Sigma); mmu-miR-135a-5p overexpression: MISSION® Lenti microRNA Human (HLMIR0200, Sigma) or control lentivirus: MISSION® TRC2 pLKO.5-puro Non-Mammalian shRNA Control (SHC202, Sigma).

Oligos used: miRCURY LNA miRNA Custom Power Inhibitor I-MMU-MIR-135A-5P and NEGATIVE CONTROL (339146, Qiagen). Mice were single-housed under standard conditions. To assess the effect of miR-135 inhibition, a first group of mice (n=4) were sacrificed 48 hours after the injection and the DG dissected for RNA extraction and miR-135 quantification. 24 hours after the

oligos injection another set of animals received 2 BrdU intraperitoneal injections per day for 5 days (50 mg/kg) (one every 12 hours). 5 mice were then sacrificed 2 hours after the last BrdU injection and the remaining 7 were sacrificed 2 weeks after the last BrdU injection (in total, 21 days after oligos injection). Brains were collected as previously described and used for RNA extraction or immunofluorescence analysis.

Dentate Gyrus extraction: Mice were sacrificed by decapitation and the brains were extracted. Immediately after extraction the brains were placed on ice-cold HBSS and the dentate gyri were micro dissected and rapidly frozen using dry ice for RNA or protein quantification.

Histology: mice were anesthetized with intraperitoneal administration of ketamine (90 mg/kg) and xylazine (5-7 mg/kg), and subsequently perfused with PBS followed by 4 % PFA. Brains were harvested, postfixed overnight in 4 % PFA, and then equilibrated in 30 % sucrose. 40 µm brain sections were generated using a sliding microtome and were stored in a −20 °C freezer as floating sections in 48 well plates filled with cryoprotectant solution (glycerol, ethylene glycol, and 0.2 M phosphate buffer, pH 7.4, 1:1:2 by volume).

6.6 Fluorescence-Activated Cell Sorting (FACS) and immunostaining analysis

FACS: For RNA extraction and cDNA preparation, six to ten Td-Tomato$^{flox/wt}$ mice were euthanized 10 or 30 days after the split cre viruses injection. DG cells were dissociated with the Neural Tissue Dissociation Kit P (Miltenyi Biotec) and FACS-sorted as previously published [205]. FACS-sorted cells were immediately processed for RNA extraction. The immunostaining on brain slices was performed on sections covering the entire dorsal hippocampus: Bregma, −1.06 to −2.18 mm [240].

Immunofluorescence: sections were washed with 0.1M PBS during 40 min and pretreated with 2N HCL at 30.2 °C for 30 min. After extensive washings with 0.1M PBS, sections were permeabilized with 0.3 % PBS-T (PBS-Triton X-100) for 10 min followed with 20 min with 0.1 % PBS-T. To detect Ki67 staining, citrate buffer 10 mM pH = 6 treatment during 10 min at 95 °C was used. Sections were blocked during 1 h with 0.1 % PBS-T and 5 % Normal Goat Serum (NGS) at RT followed by incubation with primary antibodies in a blocking solution overnight at 4 °C. The next day, after washing extensively with 0.1 % PBS-T sections were subsequently incubated for 1 h with the corresponding secondary fluorescent antibodies (1:1000; Goat Alexa 488, 568, and 647 nm, Invitrogen). Sections were counterstained with Hoechst (1:300), mounted and cover slipped with Prolong reagent. Confocal stack images of brain slices (40 µm) were ob-

Antibody	Host	Company	Catalog	Dilution
MILI	rabbit	Hannon Lab		1:100
BrdU	rat	Abcam	ab6326	1:200
KI67	rabbit	Abcam	ab15580	1:250
GFAP	rabbit	Dako	Z-0334	1:1000
RPL26	rabbit	Abcam	Ab59567	1:500
GFP	chicken	Abcam	ab13970	1:500
GLAST	rabbit	Abcam	ab416	1:200
GLT-1	rabbit	Abcam	ab41621	1:200
DCX	rabbit	Abcam	ab18723	1:1000
NEUN	mouse	Millipore	MAB377	1:250

Table 6.2: List of antibodies used for IF/IHC

tained with the Confocal A1 Nikon Inverted SFC with 20x objective. Cell quantification and analysis was performed using NIS-Elements software (Nikon) and the Cell-counter plugin in FIJI (Macbiophotonics, Fiji is Just ImageJ). For each stacked-confocal image we drew and measure the area of the DG following the position of the nuclei stained with DAPI and in that particular area we counted the positive cells. To obtain the number of cells in the volume, the density of positive cells was multiplied by the thickness of the slice (40 µm). Final cell number was corrected after checking along z-stack that no overlapping cells were counted twice.

Immuohistochemistry: 3′-DiAminoBenzidine (DAB) staining was performed as previously reported [241]. Briefly, sections were incubated with peroxidase block (Vectashield, SP-6000) for 15 min at room temperature, permeabilized with 0.3 % PBS-T for 15 min followed with 30 min with 0.1 % PBS-T. Sections were blocked during 1 h with 0.1 % PBS-T and 5 % NGS at RT, washed 3 times for 15 min with 0.1 % PBS-T and then incubated with primary antibodies in a blocking solution overnight at 4 °C. The next day, after washing extensively with 0.1 % PBS-T sections were subsequently incubated for 2 h with the corresponding biotinylated secondary antibodies (1:1000 Goat anti-rabbit, Invitrogen B2770). Signal amplification was performed using the ABC complex (Vectashield, PK-6100), according to manufacturer's instructions. Slices were washed 3 times for 15 min with PBS and then incubated with the solution for DAB reaction (Sigma, D3939). Sections were counterstained with Hoechst (1:300), mounted and cover slipped with Vectashield reagent (VECTOR Labs).

6.7 RNA extraction and Real-time PCR

Total RNA was extracted from aNPCs (proliferating and differentiating conditions), or DG dissected from adult C57BL/6 or Td-Tomato$^{flox/wt}$ mice with QIAzol protocol (Qiagen) according to the manufacturer's instructions. cDNA synthesis was obtained with ImProm-II reverse transcriptase (Promega) and was quantified with a QuantiFast SYBR Green PCR Kit (Qiagen) on a ABI-7500 Real-Time PCR System (Applied Biosystems). A 3-step PCR protocol (95 °C for 5 min, then 40 cycles of 95 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, followed by 95 °C for 15 sec and 80 °C for 15 sec) was used. Each sample was normalized to Actin levels. Expression analysis was performed using the comparative cycle threshold (Ct) method.

The primers (both forward and reverse) were designed using NCBI/UCSC Genome Browser and Primer3 software tools and then checked in PrimerBLAST for their specificity to amplify the desired genes. List of primer in the table below.

6.8 Small RNA library preparation, processing of sequencing data and piRNAs analysis

Cells are lysed in QIAzol lysis reagent and total RNA was isolated using the miRNeasy Mini kit, according to the manufacturer's instructions. Quantity and quality of the total RNA were measured by Nanodrop spectrophotometer and Experion RNA chips (Bio-Rad). RNA with RNA integrity number (RIN) values $\geq$9.5 was considered accepted for the study. 1 µg of high quality RNA for each sample was used for library preparation according to the Illumina TruSeq small RNA library protocol. Briefly, 3′ adapters were ligated to 3′ end of small RNAs using a truncated RNA ligase enzyme followed by 5′ adaptor ligation using RNA ligase enzyme. Reverse transcription followed by PCR was used to prepare cDNA using primers specific for the 3′ and 5′ adapters. The amplification of those fragments having adapter molecules on both ends was carried out with 13 PCR cycles. The amplified libraries were pooled together and run on a 6 % polyacrylamide gel. The 145-160 bp bands (which correspond to inserts of 24-32 nt cDNAs) were extracted and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The quality of the library was assessed by the Experion DNA 1K chips (Bio-Rad). Small RNA sequencing using HiSeq2000 (Illumina Inc., CA) was performed at the Center for Genomic Science of IIT@SEMM.

Processing of sequencing data: Illumina reads were trimmed to remove the 3′ adapter using Cutadapt, with parameters -m 25 -q 20. Since piRNA size ranges from 26 to 31 bases, all sequences with length $\leq$24 bases were discarded. Reads

Primer name	**Sequence** (5′-3′)
Actin Fw	GGCTGTATTCCCCTCCATCG
Actin Rv	CCAGTTGGTAACAATGCCATGT
Mili Fw	GGCCAGCATAAATCTCACAC
Mili Rv	TAGCTGGCCATCAGACACTC
Miwi Fw	TAATTGGCCTGGAGTCATCC
Miwi Rv	GAGGTAGTAGAGGCGGTTGG
Gfap Fw	GGGGCAAAAGCACCAAAGAAG
Gfap Rv	GGGACAACTTGTATTGTGAGCC
Complement C3 Fw	CCAGCTCCCCATTACGTCTG
Complement C3 Rv	GCACTTGCCTCTTTAGGAAGTC
Serpina 3n Fw	ATTTGTCCCAATGTCTGCGAA
Serpina 3n Rv	TGGCTATCTTGGCTATAAAGGGG
Cxcl10 Fw	CCAAGTGCTGCCGTCATTTTC
Cxcl10 Rv	GGCTGGCAGGGATGATTTCAA
Malat1 Fw	TAGGTTAAGTTGACGGCCGT
Malat1 Rv	CGAACTCAGAAATCCGCCTG
Vac14 Fw	AAGTGGCTCTACCATCTCTACAT
Vac14 Rv	ACAACCTCATCAGATTCGTCAGA
Lars2 Fw	CATAGAGAGGAATTTGCACCCTG
Lars 2 Rv	GCCAGTCCTGCTTCATAGAGTTT
Rpl13a Fw	AGCCTACCAGAAAGTTTGCTTAC
Rpl13a Rv	GCTTCTTCTTCCGATAGTGCATC
Rpl17 Fw	ATCAAGAGGGTCAAACCTTCGT
Rpl17 Rv	CCACCATTATACCGCCGGAA
Actin Fw	GGCTGTATTCCCCTCCATCG
Actin Rv	CCAGTTGGTAACAATGCCATGT
Ki67 Fw	ATTTCAGTTCCGCCAATCC
Ki67 Rv	GGCTTCCGTCTTCATACCTAAA
Cyclin E Fw	GATCCAGAAAAAGGAAGGCAAA
Cyclin E Rv	TGAAGAAATTGCCAAGATTGACA
Cyclin A Fw	GCCTTCACCATTCATGTGGAT
Cyclin A Rv	TTGCTGCGGGTAAAGAGACAG
Cyclin D Fw	GCGTACCCTGACACCAATCTC
Cyclin D Rv	CTCCTCTTCGCACTTCTGCTC
CDK1 Fw	CAGAGCTGGCGACCAAGAA
CDK1 Rv	GATTGACCAGCTCTTCAGGATCTT
Mcm2 Fw	ATCCACCACCGCTTCAAGAAC
Mcm2 Rv	TACCACCAAACTCTCACGGTT

Table 6.3: List of primers used for rtPCR

mapped to known non-coding RNAs (RNAcentral v6.0 Small Nucleolar RNA (snoRNA), UCSC tRNA, miRBase Release 21 miRNA hairpin and mature miRNA annotation, NCBI complete ribosomal DNA unit) [242–244] were removed from the datasets. The comparison was performed using NCBI BLASTN v2.6.0 with parameters -max_hsps= 1, -max_target_seqs= 1, -perc_identity= 80, mismatches $\leq$1, qcovhsp $\geq$90 [245]. Subsequently, the reads were aligned on the non-repeat-masked UCSC release 9 of the mouse genome (MM9) [246] using bowtie2 [247] v2.2.6 with the sensitive preset option and allowed a maximum 100 alignments. All the reads that aligned to the genome were retained and used for subsequent analysis.

DE analysis: piRNA clusters were identified collapsing overlapped piRNA sequences (piRBase Release 1) [172] into one cluster (mergeBed with preset options) [248]. piRNA clusters and all the reads that aligned to the genome were intersected (intersectBed with option -f 1). Intersection files were then parsed using a custom perl script in order to evaluate alignment counts. Differential expression was assessed using DESeq2 [249]. piRNA clusters were considered differentially expressed when the adjusted p-value was $\leq$0.05, and down- and up-regulation was established in the range of ≤ -1 to $\geq$1 log2 fold-change, respectively.

piRNA analysis: Total RNA enriched in the fraction of small RNAs, was extracted using miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions from aNPCs, DG extracted from C57BL6/J or Td-Tomato$^{flox/wt}$ mice. cDNA was obtained using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher, 4366596) according to the manufacturer's instructions and quantified using the Custom TaqMan Small RNA Assay (Thermo Fisher, 4440418) on a ABI-7500 Real-Time PCR System (Applied Biosystems). Each sample was normalized to U6 snRNA level (Thermo Fisher, 001973). Cluster sequences used for probe design are available upon request.

6.9 Mili Knock down

To constitutively downregulate mili expression in vitro, NPCs were infected at MOI=5 with a lentivirus encoding for shMili (plKO.1, Sigma), or control lentivirus (SHC202, Sigma) both decorated with an eGFP reporter. GFP-positive cells were first selected by FACS after three passages, and then plated in proliferating or differentiating media, as previously explained. For the silencing *in vivo*, 6 weeks-old mice were anesthetized with isoflurane and 1.5 µl of antisense LNA GapmeR for mili KD or negative control (Mili 339511, Control 339515, Qiagen) per DG was stereotaxically injected at the following coordinates: -2.0 anterior/posterior, ± 1.6 medial/lateral, and -1.9 to -2.1 dorsal/ventral relative to bregma (in millimeters) as previously described [169].

Mice were single-housed under standard conditions.

To assess the efficacy of mili inhibition, a first group of mice (n=5) were sacrificed 48 hours after the injection and the DG dissected for RNA or protein extraction. 24 hours after the oligos injection another set of animals received 2 BrdU intraperitoneal injections per day for 5 days (50 µg/kg) (one every 12 hours). Animals were sacrificed 10 (n=5) or 30 days after oligos injection (n=7) for histological analysis, as previously described. Mice were anesthetized with intraperitoneal administration of ketamine (90 mg/kg) and xylazine (5-7 mg/kg), and subsequently perfused with PBS followed by 4 % PFA. Brains were harvested, postfixed overnight in 4 % PFA, and then equilibrated in 30 % sucrose. µm brain sections were generated using a sliding microtome and were stored in a −20 °C freezer as floating sections in 48 well plates filled with cryoprotectant solution (glycerol, ethylene glycol, and 0.2 M phosphate buffer, pH 7.4, 1:1:2 by volume). Slices were used for immunofluorescence and immunohistochemical analysis as previously described.

6.10 *in silico* piRNA targets prediction

For piRNA targets analysis, we divided the sequencing data in one set of 100 piRNA clusters enriched in proliferating aNPCs (DIV0) and a second set of 198 clusters specifically expressed at DIV4/7 stage. The Differential Expression analysis for piRNAs mapping on REs showing trends for enrichment of piRNAs mapping on repeat elements (REs) in DIV4 and DIV7 compared to DIV0 was done using EdgeR software package [250].

Identification of piRNA targets was divided in: piRNAs mapping on REs only / piRNAs mapping on GENCODE elements / piRNAs mapping on REs within GENCODE elements / unannotated piRNAs / piRNAs clusters. Gene Ontology analysis for piRNAs mapping on GENCODE protein-coding genes (but NOT mapping on REs) has been done with the R package GOFuncR (https://bioconductor.org/packages/release/bioc/html/GOfuncR.html).

6.11 Kainic acid administration, single cell suspension and enrichment of NSC using FACS

Kainic Acid was administered as described before [241]. Briefly, 50 nl of 2.22mM Kainic Acid dissolved in PBS (pH 7.4) was injected bilaterally into the hippocampus at the following coordinates (AP −2.0, ML ±1.5, DV −2.0 mm) (between 9AM and 1PM) to elicit tonic, non-convulsive epileptic seizures reaching a max score of 3 on the Racine scale. Control animals were administered saline (pH 7.4).

Single Cell Suspension: Bilateral dentate gyri from 3 animals per condition were pooled to allow sufficient recovery of NPCs. A single cell suspension was created using a Neural Tissue Dissociation kit (Miltenyi Biotec), according to the manufacturers protocol. Briefly, tissue was dissociated and the single cell suspension was passed through a 40 µm filter and DNAse was added (1250 units/mL). The suspensions were centrifuged at 300xG for 11 min, and the cell pellet was resuspended in 50 µl PBS containing 1 % BSA and kept on ice.

Enrichment of Neural Stem and Progenitor Cells using FACS: In order to enrich aNPCs from the DG, we made use of the endogenous GFP expression driven by the Nestin promotor in combination with FACS. Propium Iodide (5 µg/ml) was added to the single cell suspension to assess cell viability. Cells were sorted using a FACSAriatm III system (BD) with 488 nm excitation laser. Propium Iodide was detected within the PE/Texas Red channel with a 610/20 bandpass filter and GFP within the FITC channel with a 530/30 band pass filter. Cell duplets were removed based on forward and side scatter and viable cells were selected based on propium iodide negativity. GFP-positive (corrected for autofluorescence) cells were sorted (50000 cells/pool) and collected in PBS containing 1 % FBS. After collection, the NPC-enriched cell suspensions were spun down at 300xG for 11 min. Supernatants were removed, leaving approximately 250 µl of 1 % FBS-PBS. 0.75 ml Trizol LS (Thermo Scientific) was added and after resuspension samples were snap-frozen and stored at −20 °C.

6.12 Total RNA and soluble proteins measurement

To compare protein content in control and Mili KD aNPCs, we plated the GFP+ cells sorted after lentiviral transduction (scrambled control and Mili KD) in proliferating media; after 48 hours we collected the cells using the StemPro Accutase (Gibco, A1110501), we resuspend the pellet in PBS and we divided it by FACS in three fractions containing the same number of cells (600k). We proceeded with DNA, RNA and protein extraction from the fractions of control and Mili KD samples (n=7).

Genomic DNA was extracted using the DNeasy Blood & Tissue Kits (Qiagen, 69504) according to the the manufacturer's instructions and quantified by Nanodrop spectrophotometer. Total RNA was extracted from cell pellets using the RNeasy Micro Kit (Qiagen, 74004) according to the manufacturer's instructions and quantified by Nanodrop spectrophotometer. Soluble proteins were extracted from cells using RIPA buffer and the concentration was measured using the Bradford Assay kit (Bio-Rad). We normalized the amount of RNA and protein on the number of cells counted by the FACS.

6.13 Immunofluorescence, STED nanoscopy and particle analysis

aNPCs were plated on glass coverslips 24 h before fixation. Cells were fixed with PFA 4 %, permeabilized with PBS-Triton 0.1 %, blocked 1 hour at room temperature with PBS-T 0.1 % NGS 5 % and incubated according to the dilution suggested by the manufacturer's instructions with 0.01 µg/ml rabbit polyclonal antibody against the N terminus of RPL26 (Abcam) for 1 h at room temperature. Cells were washed extensively and incubated with the secondary antibody goat anti–rabbit ATTO-488 (0.005–1 µg/ml; Sigma-Aldrich) for 45 min. Nuclei were stained while mounting the coverslip with DAPI-Prolong antifade (Invitrogen).

Confocal and STED images were acquired at 23 °C with a TCS SP5 STED gated (Leica) operated with Leica's microscope imaging software. All of the images have 14 nm pixel size and 37 µs pixel dwell time. The ATTO-488 fluorescence was excited at 488 nm by means of a supercontinuum- pulsed laser system, and the fluorescence depletion was performed by a 592 nm cw-laser beam. The maximal focal power of the STED beam was 120 mW. Both beams were focused into the 1.4 NA objective lens (HCX PL APO 100x1.40 NA Oil STED Orange; Leica). Fluorescence was collected by the same lens, filtered with a 592 notch filter, and imaged in the spectral range 500–550 nm by hybrid detector with a time gating of 1.5 ns. We performed the analysis of polysome clusters in aNPCs over 26 images of 16 different cells. Image analysis was performed using the Fiji software.

6.14 *In Situ* Hybridization

Mice were anesthetized and perfused transcardially with cold 4 % paraformaldehyde in 1x PBS. Brains were removed and post-fixed in the same fixative for 5 hours at 4 °C. Tissues were washed several times in 1x PBS prior to dehydration with 30 % sucrose in 1x PBS, overnight (or until they sink) at 4 °C and carefully dried before proceeding with the flash freezing protocol: tissues were placed in a metal beaker filled with isopentane (Sigma), located in a foam cooler or laboratory ice bucket and surrounded with crushed dry ice. 18-20 µm brain slices were collected using a cryostat and attached on glass slides. Slices were permeabilized by treating twice for 10 min with RIPA buffer (NaCl 150 mM, NP-40 1 %, Na deoxycholate 0.5 %, Sodium Dodecyl Sulfate (SDS) 0.1 %, EDTA 1 mM, Tris pH 8.0 50 mM) and post-fixed for 10 min in 4 % PFA in 1x PBS, followed by washes in 1X PBS to remove the excess PFA. The positive charges in the tissue were blocked by treating slides for 15 min with acetic anhydride (Sigma) (0.25 % final concentration) in triethanolamine buffer (tri-

ethanolamine 100 mM, acetic acid pH 8.0), followed by washes with 1x PBS.

In order to block aspecific sites, a pre-hybridization step with 200-250 µl hybridization solution (Formamide 50 %, SSC 5X, Denhardts 5x, Salmon sperm DNA (500 µg/ml, yeast RNA 250 µg/ml) was carried out. After 1 hour, a hybridization solution containing 160 nM (miR-135, miR190, miR203) or 100 nM (miR9) of the DIG-labeled LNA probe (Exiqon) was added and incubated ON. The next day, slides were washed for 1 hour with a post-hybridization solution (Formamide 50 %, SSC 2X, Tween-20 0.1 %). For the immunological detection, slides were incubated twice for 5 min and once for 20 min at RT in buffer B1 (Maleic acid 100 mM pH 7.5, NaCl 100mM, Tween-20 0.1 %) and then blocked in buffer B2 (10 % Normal Goat Serum in B1) for 1 hour. The anti-DIG antibody (Roche) was diluted 1:2000 in B2 buffer and incubated ON at 4 °C. For development of the color reactions, two different alkaline phosphatase substrates were used: NBT/BCIP (Roche) or Fast Red TR/Naphthol AS-MX solution (Sigma), according to manufacturer instructions. The reaction was stopped by several washes with 0.1 % Tween 20 in 1x PBS. Sections were mounted using mounting resins (Thermo Fisher) or in VECTA-Shield mounting medium (VECTOR Labs) including DAPI, and imaged using conventional bright-field microscopy or a confocal microscope with the Cy-3 filter.

6.15 Proteomics

NPCs (three independent experiments) were lysed with RIPA buffer, and 50 mg of proteins was collected from all the samples and processed as previously described [251]. Protein pools were processed for liquid chromatography-tandem mass spectrometry analysis.

6.16 Statistical analysis

Data are presented as mean pmSEM and were analyzed using Prism 6 (GraphPad). Statistical significance was assessed with a two-tailed unpaired t test for two experimental groups. For experiments with three or more groups, one-way ANOVA with Bonferroni's multiple comparison test as post hoc was used. Results were considered significant when $p<0.05$.

Bibliography

[1] M. Pons-Espinal, C. Gasperini, M. J. Marzi, C. Braccia, A. Armirotti, A. Pötzsch, T. L. Walker, K. Fabel, F. Nicassio, G. Kempermann, D. De Pietri Tonelli, MiR-135a-5p Is Critical for Exercise-Induced Adult Neurogenesis. Stem Cell Reports. 12, 1298–1312 (2019).

[2] J. Altman, Are new neurons formed in the brains of adult mammals? Science. 135, 1127–1128 (1962).

[3] P. Rakic, R. S. Nowakowski, The time of origin of neurons in the hippocampal region of the rhesus monkey. J. Comp. Neurol. 196, 99–128 (1981).

[4] P. S. Eriksson, E. Perfilieva, T. Björk-Eriksson, A.-M. Alborn, C. Nordborg, D. A. Peterson, F. H. Gage, Neurogenesis in the adult human hippocampus. Nat. Med. 4, 1313–1317 (1998).

[5] K. L. Spalding, R. D. Bhardwaj, B. A. Buchholz, H. Druid, J. Frisén, Retrospective birth dating of cells in humans. Cell. 122, 133–143 (2005).

[6] G. Kempermann, H. Song, F. H. Gage, Neurogenesis in the Adult Hippocampus. Cold Spring Harb. Perspect. Biol. 7, a018812 (2015).

[7] L. Peng, M. A. Bonaguidi, Function and Dysfunction of Adult Hippocampal Neurogenesis in Regeneration and Disease. Am. J. Pathol. 188, 23–28 (2018).

[8] A. M. Bond, G. L. Ming, H. Song, Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell. 17, 385–395 (2015).

[9] Z. Nicola, K. Fabel, G. Kempermann, Development of the adult neurogenic niche in the hippocampus of mice. Front. Neuroanat. 9, 53 (2015).

[10] P. Bielefeld, C. Mooney, D. C. Henshall, C. P. Fitzsimons, miRNA-Mediated Regulation of Adult Hippocampal Neurogenesis; Implications for Epilepsy. Brain Plast. 3, 43–59 (2016).

[11] H. G. Kuhn, M. Biebl, D. Wilhelm, M. Li, R. M. Friedlander, J. Winkler, Increased generation of granule cells in adult Bcl-2-overexpressing mice: A role for cell death during continued hippocampal neurogenesis. Eur. J. Neurosci. 22, 1907–1915 (2005).

[12] M. Biebl, C. M. Cooper, J. Winkler, H. G. Kuhn, Analysis of neurogenesis and programmed cell death reveals a self- renewing capacity in the adult rat brain. Neurosci. Lett. 291, 17–20 (2000).

[13] G. Kempermann, S. Jessberger, B. Steiner, G. Kronenberg, Milestones of neuronal development in the adult hippocampus. Trends Neurosci. 27, 447–452 (2004).

[14] G. li Ming, H. Song, Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. Neuron. 70 (2011), pp. 687–702.

[15] A. Pino, G. Fumagalli, F. Bifari, I. Decimo, New neurons in adult brain: distribution, molecular mechanisms and therapies. Biochem. Pharmacol. 141, 4–22 (2017).

[16] N. S. Roy, S. Wang, L. Jiang, J. Kang, A. Benraiss, C. Harrison-Restelli, R. A. R. Fraser, W. T. Couldwell, A. Kawaguchi, H. Okano, M. Nedergaard, S. A. Goldman, In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat. Med. 6, 271–277 (2000).

[17] M. C. Nunes, N. S. Roy, H. M. Keyoung, R. R. Goodman, G. McKhann, L. Jiang, J. Kang, M. Nedergaard, S. A. Goldman, Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. Nat. Med. 9, 439–447 (2003).

[18] J. M. Encinas, T. V. Michurina, N. Peunova, J. H. Park, J. Tordo, D. A. Peterson, G. Fishell, A. Koulakov, G. Enikolopov, Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. Cell Stem Cell. 8, 566–579 (2011).

[19] N. Sanai, T. Nguyen, R. A. Ihrie, Z. Mirzadeh, H.-H. Tsai, M. Wong, N. Gupta, M. S. Berger, E. Huang, J.-M. Garcia-Verdugo, D. H. Rowitch, A. Alvarez-Buylla, Corridors of migrating neurons in the human brain and their decline during infancy. Nature. 478, 382–386 (2011).

[20] K. L. Spalding, O. Bergmann, K. Alkass, S. Bernard, M. Salehpour, H. B. Huttner, E. Boström, I. Westerlund, C. Vial, B. A. Buchholz, G. Possnert, D. C. Mash, H. Druid, J. Frisén, Dynamics of Hippocampal Neurogenesis in Adult Humans. Cell. 153, 1219–1227 (2013).

[21] M. Boldrini, C. A. Fulmore, A. N. Tartt, L. R. Simeon, I. Pavlova, V. Poposka, G. B. Rosoklija, A. Stankov, V. Arango, A. J. Dwork, R. Hen, J. J. Mann, Human Hippocampal Neurogenesis Persists throughout Aging. Cell Stem Cell. 22, 589-599.e5 (2018).

[22] J. T. Gonçalves, S. T. Schafer, F. H. Gage, Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. Cell. 167, 897–914 (2016).

[23] G. Kempermann, Activity dependency and aging in the regulation of adult neurogenesis. Cold Spring Harb. Perspect. Biol. 7 (2015), doi:10.1101/cshperspect.a018929.

[24] B. L. Jacobs, H. Van Praag, F. H. Gage, Adult brain neurogenesis and psychiatry: A novel theory of depression. Mol. Psychiatry. 5 (2000), pp. 262–269.

[25] T. D. Ardizzone, X. Zhan, B. P. Ander, F. R. Sharp, Src kinase inhibition improves acute outcomes after experimental intracerebral hemorrhage. Stroke. 38, 1621–1625 (2007).

[26] X. Wang, X. Gao, S. Michalski, S. Zhao, J. Chen, Traumatic Brain Injury Severity Affects Neurogenesis in Adult Mouse Hippocampus. J. Neurotrauma. 33, 721–733 (2016).

[27] T. Toda, F. H. Gage, Review: adult neurogenesis contributes to hippocampal plasticity. Cell Tissue Res. 373, 693–709 (2018).

[28] M. A. Bonaguidi, J. Song, G. Ming, H. Song, A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. Curr. Opin. Neurobiol. 22, 754–761 (2012).

[29] G. Kempermann, Seven principles in the regulation of adult neurogenesis. Eur. J. Neurosci. 33, 1018–1024 (2011).

[30] M. W. Marlatt, P. J. Lucassen, Neurogenesis and Alzheimer's disease: Biology and pathophysiology in mice and men. Curr. Alzheimer Res. 7, 113–25 (2010).

[31] M. Pons-Espinal, M. M. de Lagran, M. Dierssen, Functional implications of hippocampal adult neurogenesis in intellectual disabilities. Amino Acids. 45, 113–131 (2013).

[32] P. Dibajnia, C. M. Morshead, Role of neural precursor cells in promoting repair following stroke. Acta Pharmacol. Sin. 34, 78–90 (2013).

[33] F. Doetsch, L. Petreanu, I. Caille, J. M. Garcia-Verdugo, A. Alvarez-Buylla, EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron. 36, 1021–34 (2002).

[34] A. Sierra, S. Martín-Suárez, R. Valcárcel-Martín, J. Pascual-Brazo, S.-A. Aelvoet, O. Abiega, J. J. Deudero, A. L. Brewster, I. Bernales, A. E. Anderson, V. Baekelandt, M. Maletić-Savatić, J. M. Encinas, Neuronal Hyperactivity Accelerates Depletion of Neural Stem Cells and Impairs Hippocampal Neurogenesis. Cell Stem Cell. 16, 488–503 (2015).

[35] R. Beckervordersandforth, C.-L. Zhang, D. C. Lie, Transcription-Factor-Dependent Control of Adult Hippocampal Neurogenesis. Cold Spring Harb. Perspect. Biol. 7, a018879 (2015).

[36] S. E. Castel, R. A. Martienssen, RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nat. Rev. Genet. 14, 100–112 (2013).

[37] F. M. Cernilogar, M. C. Onorati, G. O. Kothe, A. M. Burroughs, K. M. Parsi, A. Breiling, F. Lo Sardo, A. Saxena, K. Miyoshi, H. Siomi, M. C. Siomi, P. Carninci, D. S. Gilmour, D. F. V. Corona, V. Orlando, Chromatin-associated RNA interference components contribute to transcriptional regulation in Drosophila. Nature. 480, 391–395 (2011).

[38] V. Huang, L.-C. Li, Demystifying the nuclear function of Argonaute proteins. RNA Biol. 11, 18–24 (2014).

[39] H. Noguchi, A. Kimura, N. Murao, T. Matsuda, M. Namihira, K. Nakashima, Expression of DNMT1 in neural stem/precursor cells is critical for survival of newly generated neurons in the adult hippocampus. Neurosci. Res. 95, 1–11 (2015).

[40] M. Schouten, S. A. Fratantoni, C. J. Hubens, S. R. Piersma, T. V. Pham, P. Bielefeld, R. A. Voskuyl, P. J. Lucassen, C. R. Jimenez, C. P. Fitzsimons, MicroRNA-124 and -137 cooperativity controls caspase-3 activity through BCL2L13 in hippocampal neural stem cells. Sci. Rep. 5, 12448 (2015).

[41] Wu, C.T., and Morris, J.R. (2001). Genes, genetics, and epigenetics: A correspondence. Science (80-.). 293, 1103–1105.

[42] J. Hsieh, X. Zhao, Genetics and epigenetics in adult neurogenesis. Cold Spring Harb. Perspect. Biol. 8 (2016), doi:10.1101/cshperspect.a018911.

[43] Hsieh, J., Nakashima, K., Kuwabara, T., Mejia, E., and Gage, F.H. (2004). Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. Proc. Natl. Acad. Sci. U. S. A. 101, 16659–16664.

[44] Sun, G., Alzayady, K., Stewart, R., Ye, P., Yang, S., Li, W., and Shi, Y. (2010). Histone demethylase LSD1 regulates neural stem cell proliferation. Mol. Cell. Biol. 30, 1997–2005.

[45] Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. Nat. Genet. 33, 245–254.

[46] Mohn, F., Weber, M., Rebhan, M., Roloff, T.C., Richter, J., Stadler, M.B., Bibel, M., and Schübeler, D. (2008). Lineage-Specific Polycomb Targets and De Novo DNA Methylation Define Restriction and Potential of Neuronal Progenitors. Mol. Cell 30, 755–766.

[47] Feng, J., Chang, H., Li, E., and Fan, G. (2005). Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. J. Neurosci. Res. 79, 734–746.

[48] Feng, J., Zhou, Y., Campbell, S.L., Le, T., Li, E., Sweatt, J.D., Silva, A.J., and Fan, G. (2010). Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. Nat. Neurosci. 13, 423–430.

[49] Levenson, J.M., Roth, T.L., Lubin, F.D., Miller, C.A., Huang, I.C., Desai, P., Malone, L.M., and Sweatt, J.D. (2006). Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. J. Biol. Chem. 281, 15763–15773.

[50] Fan, G., Martinowich, K., Chin, M.H., He, F., Fouse, S.D., Hutnick, L., Hattori, D., Ge, W., Shen, Y., Wu, H., et al. (2005). DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 132, 3345–3356.

[51] O. Wapinski, H. Y. Chang, Long noncoding RNAs and human disease. Trends Cell Biol. 21 (2011), pp. 354–361.

[52] S. Y. Ng, L. Lin, B. S. Soh, L. W. Stanton, Long noncoding RNAs in development and disease of the central nervous system. Trends Genet. 29 (2013), pp. 461–468.

[53] K. C. Wang, H. Y. Chang, Molecular Mechanisms of Long Noncoding RNAs. Mol. Cell. 43 (2011), pp. 904–914.

[54] T. R. Mercer, M. E. Dinger, S. M. Sunkin, M. F. Mehler, J. S. Mattick, Specific expression of long noncoding RNAs in the mouse brain. Proc. Natl. Acad. Sci. U. S. A. 105, 716–721 (2008).

[55] V. K. Gangaraju, H. Lin, MicroRNAs: Key regulators of stem cells. Nat. Rev. Mol. Cell Biol. 10 (2009), pp. 116–125.

[56] T. A. Farazi, S. A. Juranek, T. Tuschl, The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. Development. 135, 1201–1214 (2008).

[57] L. Wu, J. G. Belasco, Let Me Count the Ways: Mechanisms of Gene Regulation by miRNAs and siRNAs. Mol. Cell. 29, 1–7 (2008).

[58] T. Thomson, H. Lin, The Biogenesis and Function of PIWI Proteins and piRNAs: Progress and Prospect. Annu. Rev. Cell Dev. Biol. 25, 355–376 (2009).

[59] S. T. Grivna, E. Beyret, Z. Wang, H. Lin, RESEARCH COMMUNICATION A novel class of small RNAs in mouse spermatogenic cells. Genes Dev. 20, 1709–1714 (2006).

[60] T. Hirano, Y. W. Iwasaki, Z. Y. C. Lin, M. Imamura, N. M. Seki, E. Sasaki, K. Saito, H. Okano, M. C. Siomi, H. Siomi, Small RNA profiling and characterization of piRNA clusters in the adult testes of the common marmoset, a model primate. Rna. 20, 1223–1237 (2014).

[61] A. Aravin, D. Gaidatzis, S. Pfeffer, M. Lagos-Quintana, P. Landgraf, N. Iovino, P. Morris, M. J. Brownstein, S. Kuramochi-Miyagawa, T. Nakano, M. Chien, J. J. Russo, J. Ju, R. Sheridan, C. Sander, M. Zavolan, T. Tuschl, A novel class of small RNAs bind to MILI protein in mouse testes. Nature. 442, 203–207 (2006).

[62] C. Juliano, J. Wang, H. Lin, Uniting Germline and Stem Cells: The Function of Piwi Proteins and the piRNA Pathway in Diverse Organisms. Annu. Rev. Genet. 45, 447–469 (2011).

[63] G. J. Hannon, F. V. Rivas, E. P. Murchison, J. A. Steitz, The expanding universe of noncoding RNAs. Cold Spring Harb. Symp. Quant. Biol. 71, 551–564 (2006).

[64] G. Stefani, F. J. Slack, Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol. 9, 219–230 (2008).

[65] Y.-K. Kim, V. N. Kim, Processing of intronic microRNAs. EMBO J. 26, 775–783 (2007).

[66] D. de Rie, I. Abugessaisa, T. Alam, E. Arner, P. Arner, H. Ashoor, G. Åström, M. Babina, N. Bertin, A. M. Burroughs, A. J. Carlisle, C. O.

Daub, M. Detmar, R. Deviatiiarov, A. Fort, C. Gebhard, D. Goldowitz, S. Guhl, T. J. Ha, J. Harshbarger, A. Hasegawa, K. Hashimoto, M. Herlyn, P. Heutink, K. J. Hitchens, C. C. Hon, E. Huang, Y. Ishizu, C. Kai, T. Kasukawa, P. Klinken, T. Lassmann, C.-H. Lecellier, W. Lee, M. Lizio, V. Makeev, A. Mathelier, Y. A. Medvedeva, N. Mejhert, C. J. Mungall, S. Noma, M. Ohshima, M. Okada-Hatakeyama, H. Persson, P. Rizzu, F. Roudnicky, P. Sætrom, H. Sato, J. Severin, J. W. Shin, R. K. Swoboda, H. Tarui, H. Toyoda, K. Vitting-Seerup, L. Winteringham, Y. Yamaguchi, K. Yasuzawa, M. Yoneda, N. Yumoto, S. Zabierowski, P. G. Zhang, C. A. Wells, K. M. Summers, H. Kawaji, A. Sandelin, M. Rehli, Y. Hayashizaki, P. Carninci, A. R. R. Forrest, M. J. L. de Hoon, M. J. L. de Hoon, An integrated expression atlas of miRNAs and their promoters in human and mouse. Nat. Biotechnol. 35, 872–878 (2017).

[67] W. Xu, A. San Lucas, Z. Wang, Y. Liu, Identifying microRNA targets in different gene regions. BMC Bioinformatics. 15, S4 (2014).

[68] L. P. Lim, N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, J. M. Johnson, Microarray analysis shows that some microRNAs downregulate large numbers of-target mRNAs. Nature. 433, 769–773 (2005).

[69] M. Selbach, B. Schwanhäusser, N. Thierfelder, Z. Fang, R. Khanin, N. Rajewsky, Widespread changes in protein synthesis induced by microRNAs. Nature. 455, 58–63 (2008).

[70] M. Ha, V. N. Kim, Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. 15, 509–524 (2014).

[71] V. Olive, A. C. Minella, L. He, Outside the coding genome, mammalian microRNAs confer structural and functional complexity. Sci. Signal. 8 (2015), p. re2.

[72] L. Stappert, F. Klaus, O. Brüstle, MicroRNAs Engage in Complex Circuits Regulating Adult Neurogenesis. Front. Neurosci. 12, 707 (2018).

[73] J. G. Ruby, C. H. Jan, D. P. Bartel, Intronic microRNA precursors that bypass Drosha processing. Nature. 448, 83–86 (2007).

[74] J. E. Babiarz, J. G. Ruby, Y. Wang, D. P. Bartel, R. Blelloch, Mouse ES cells express endogenous shRNAs, siRNAs, and other microprocessor-independent, dicer-dependent small RNAs. Genes Dev. 22, 2773–2785 (2008).

[75] D. Cifuentes, H. Xue, D. W. Taylor, H. Patnode, Y. Mishima, S. Cheloufi, E. Ma, S. Mane, G. J. Hannon, N. D. Lawson, S. A. Wolfe, A. J. Giraldez, A novel miRNA processing pathway independent of dicer requires argonaute2 catalytic activity. Science (80-.). 328, 1694–1698 (2010).

[76] S. Cheloufi, C. O. Dos Santos, M. M. W. Chong, G. J. Hannon, A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. Nature. 465, 584–589 (2010).

[77] R. Petri, J. Malmevik, L. Fasching, M. Åkerblom, J. Jakobsson, miRNAs in brain development. Exp. Cell Res. (2013), doi:10.1016/j.yexcr.2013.09.022.

[78] F. Marinaro, M. J. Marzi, N. Hoffmann, H. Amin, R. Pelizzoli, F. Niola, F. Nicassio, D. De Pietri Tonelli, MicroRNA-independent functions of DGCR8 are essential for neocortical development and TBR1 expression. EMBO Rep. 18, 603–618 (2017).

[79] S. R. Heras, S. Macias, M. Plass, N. Fernandez, D. Cano, E. Eyras, J. L. Garcia-Perez, J. F. Cáceres, The Microprocessor controls the activity of mammalian retrotransposons. Nat. Struct. Mol. Biol. 20, 1173–1181 (2013).

[80] N. Hoffmann, S. C. Weise, F. Marinaro, T. Vogel, D. De Pietri Tonelli, DGCR8 promotes neural progenitor expansion and represses neurogenesis in the mouse embryonic neocortex. Front. Neurosci. 12 (2018), doi:10.3389/fnins.2018.00281.

[81] X. Cao, G. Yeo, A. R. Muotri, T. Kuwabara, F. H. Gage, NONCODING RNAS IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM. Annu. Rev. Neurosci. 29, 77–103 (2006).

[82] A. M. KRICHEVSKY, K. S. King, C. P. Donahue, K. Khrapko, K. S. Kosik, A microRNA array reveals extensive regulation of microRNAs during brain development. RNA. 9, 1274–1281 (2003).

[83] E. A. Miska, E. Alvarez-Saavedra, M. Townsend, A. Yoshii, N. Šestan, P. Rakic, M. Constantine-Paton, H. R. Horvitz, Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol. 5, R68 (2004).

[84] L. F. Sempere, S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky, V. Ambros, Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 5, R13 (2004).

[85] C. Conaco, S. Otto, J.-J. Han, G. Mandel, Reciprocal actions of REST and a microRNA promote neuronal identity. Proc. Natl. Acad. Sci. 103, 2422–2427 (2006).

[86] A. M. Krichevsky, K.-C. Sonntag, O. Isacson, K. S. Kosik, Specific MicroRNAs Modulate Embryonic Stem Cell-Derived Neurogenesis. Stem Cells. 24, 857–864 (2006).

[87] E. V. Makeyev, J. Zhang, M. A. Carrasco, T. Maniatis, The MicroRNA miR-124 Promotes Neuronal Differentiation by Triggering Brain-Specific Alternative Pre-mRNA Splicing. Mol. Cell. 27, 435–448 (2007).

[88] L. Smirnova, A. Gräfe, A. Seiler, S. Schumacher, R. Nitsch, F. G. Wulczyn, Regulation of miRNA expression during neural cell specification. Eur. J. Neurosci. 21, 1469–1477 (2005).

[89] O. Barca-Mayo, D. De Pietri Tonelli, Convergent microRNA actions coordinate neocortical development. Cell. Mol. Life Sci. 71, 2975–2995 (2014).

[90] D. De Pietri Tonelli, J. N. Pulvers, C. Haffner, E. P. Murchison, G. J. Hannon, W. B. Huttner, miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. Development. 135, 3911–3921 (2008).

[91] N. Murao, H. Noguchi, K. Nakashima, Epigenetic regulation of neural stem cell property from embryo to adult. Neuroepigenetics. 5, 1–10 (2016).

[92] M. A. Lopez-Ramirez, S. Nicoli, Role of mirnas and epigenetics in neural stem cell fate determination. Epigenetics. 9, 90–100 (2014).

[93] P. Bielefeld, A. Sierra, J. M. Encinas, M. Maletic-Savatic, A. Anderson, C. P. Fitzsimons, A Standardized Protocol for Stereotaxic Intrahippocampal Administration of Kainic Acid Combined with Electroencephalographic Seizure Monitoring in Mice. Front. Neurosci. 11 (2017), doi:10.3389/fnins.2017.00160.

[94] S. E. Khoshnam, W. Winlow, M. Farzaneh, Y. Farbood, H. F. Moghaddam, Pathogenic mechanisms following ischemic stroke. Neurol. Sci. 38 (2017), pp. 1167–1186.

[95] L. Qiu, E. K. Tan, L. Zeng, in Advances in Experimental Medicine and Biology (Springer New York LLC, 2015), vol. 888, pp. 51–70.

[96] J. M. Encinas, C. P. Fitzsimons, Gene regulation in adult neural stem cells. Current challenges and possible applications. Adv. Drug Deliv. Rev. 120, 118–132 (2017).

[97] M. Pons-Espinal, E. de Luca, M. J. Marzi, R. Beckervordersandforth, A. Armirotti, F. Nicassio, K. Fabel, G. Kempermann, D. De Pietri Tonelli, Synergic Functions of miRNAs Determine Neuronal Fate of Adult Neural Stem Cells. Stem Cell Reports. 8, 1046–1061 (2017).

[98] M. Schouten, A. Aschrafi, P. Bielefeld, E. Doxakis, C. P. Fitzsimons, MicroRNAs and the regulation of neuronal plasticity under stress conditions. Neuroscience. 241 (2013), pp. 188–205.

[99] Y. M. Clovis, W. Enard, F. Marinaro, W. B. Huttner, D. de Pietri Tonelli, Convergent repression of Foxp2 3′UTR by miR-9 and miR-132 in embryonic mouse neocortex: Implications for radial migration of neurons. Dev. 139, 3332–3342 (2012).

[100] C. Zhao, G. Sun, S. Li, Y. Shi, A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. Nat. Struct. Mol. Biol. 16, 365–371 (2009).

[101] C. Zhao, G. Sun, S. Li, M. F. Lang, S. Yang, W. Li, Y. Shia, MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. Proc. Natl. Acad. Sci. U. S. A. 107, 1876–1881 (2010).

[102] S. Bian, T. le Xu, T. Sun, Tuning the cell fate of neurons and glia by microRNAs. Curr. Opin. Neurobiol. 23 (2013), pp. 928–934.

[103] S. Arora, R Rana, A Chhabra, A Jaiswal, V Rani, miRNA-transcription factor interactions: a combinatorial regulation of gene expression, doi:10.1007/s00438-013-0734-z.

[104] M. Osella, A. Riba, A. Testori, D. Corá, M. Caselle, Interplay of microRNA and epigenetic regulation in the human regulatory network. Front. Genet. 5 (2014), doi:10.3389/fgene.2014.00345.

[105] A. S. Yoo, A. X. Sun, L. Li, A. Shcheglovitov, T. Portmann, Y. Li, C. Lee-Messer, R. E. Dolmetsch, R. W. Tsien, G. R. Crabtree, MicroRNA-mediated conversion of human fibroblasts to neurons. Nature. 476 (2011), pp. 228–231.

[106] D. N. Cox, A. Chao, J. Baker, L. Chang, D. Qiao, H. Lin, A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. 12, 3715–3727 (1998).

[107] C. Li, V. V. Vagin, S. Lee, J. Xu, S. Ma, H. Xi, H. Seitz, M. D. Horwich, M. Syrzycka, B. M. Honda, E. L. W. Kittler, M. L. Zapp, C. Klattenhoff, N. Schulz, W. E. Theurkauf, Z. Weng, P. D. Zamore, Collapse of Germline piRNAs in the Absence of Argonaute3 Reveals Somatic piRNAs in Flies. Cell. 137, 509–521 (2009).

[108] H. Lin, A. C. Spradling, A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. Development. 124, 2463–2476 (1997).

[109] E. F. Roovers, D. Rosenkranz, M. Mahdipour, C. T. Han, N. He, S. M. C. de Sousa Lopes, L. A. J. van der Westerlaken, H. Zischler, F. Butter, B. A. J. Roelen, R. F. Ketting, Piwi proteins and piRNAs in Mammalian Oocytes and early embryos. Cell Rep. 10, 2069–2082 (2015).

[110] M. A. Carmell, A. Girard, H. J. G. van de Kant, D. Bourc'his, T. H. Bestor, D. G. de Rooij, G. J. Hannon, MIWI2 Is Essential for Spermatogenesis and Repression of Transposons in the Mouse Male Germline. Dev. Cell. 12, 503–514 (2007).

[111] W. Deng, H. Lin, N. Carolina, miwi , a Murine Homolog of piwi , Encodes a Cytoplasmic Protein Essential for Spermatogenesis. 2, 819–830 (2002).

[112] S. Kuramochi-Miyagawa, Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. Development (2004), doi:10.1242/dev.00973.

[113] X. Ding, H. Guan, H. Li, Characterization of a piRNA binding protein Miwi in mouse oocytes. Theriogenology. 79 (2013), doi:10.1016/j.theriogenology.2012.11.013.

[114] A. K. Lim, C. Lorthongpanich, T. G. Chew, C. W. G. Tan, Y. T. Shue, S. Balu, N. Gounko, S. Kuramochi-Miyagawa, M. M. Matzuk, S. Chuma, D. M. Messerschmidt, D. Solter, B. B. Knowles, The nuage mediates retrotransposon silencing in mouse primordial ovarian follicles. Dev. 140, 3819–3825 (2013).

[115] J. H. Lee, D. Schütte, G. Wulf, L. Füzesi, H. J. Radzun, S. Schweyer, W. Engel, K. Nayernia, Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XLpathway. Hum. Mol. Genet. 15, 201–211 (2006).

[116] D. Qiao, A. M. Zeeman, W. Deng, L. H. J. Looijenga, H. Lin, Molecular characterization of hiwi, a human member of the piwi gene family whose

overexpression is correlated to seminomas. Oncogene. 21, 3988–3999 (2002).

[117] X. Liu, Y. Sun, J. Guo, H. Ma, J. Li, B. Dong, G. Jin, J. Zhang, J. Wu, L. Meng, C. Shou, Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells. Int. J. Cancer. 118, 1922–1929 (2006).

[118] D. N. Cox, A. Chao, H. Lin, piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development. 127, 503–514 (2000).

[119] H. B. Megosh, D. N. Cox, C. Campbell, H. Lin, The Role of PIWI and the miRNA Machinery in Drosophila Germline Determination. Curr. Biol. 16, 1884–1894 (2006).

[120] K. Saito, Y. Sakaguchi, T. Suzuki, T. Suzuki, H. Siomi, M. C. Siomi, Pimet, the Drosophila homolog of HEN1, mediates 2′-O-methylation of Piwi-interacting RNAs at their 3′ ends. Genes Dev. 21, 1603–1608 (2007).

[121] Y. W. Iwasaki, M. C. Siomi, H. Siomi, PIWI-Interacting RNA: Its Biogenesis and Functions. Annu. Rev. Biochem. 84, 405–433 (2015).

[122] C. D. Malone, G. J. Hannon, Small RNAs as Guardians of the Genome. Cell. 136 (2009), pp. 656–668.

[123] H. Ishizu, H. Siomi, M. C. Siomi, Biology of Piwi-interacting RNAs: New insights into biogenesis and function inside and outside of germlines. Genes Dev. 26 (2012), pp. 2361–2373.

[124] R. J. Ross, M. M. Weiner, H. Lin, PIWI proteins and PIWI-interacting RNAs in the soma. Nature. 505 (2014), pp. 353–359.

[125] C. Klattenhoff, D. P. Bratu, N. McGinnis-Schultz, B. S. Koppetsch, H. A. Cook, W. E. Theurkauf, Drosophila rasiRNA Pathway Mutations Disrupt Embryonic Axis Specification through Activation of an ATR/Chk2 DNA Damage Response. Dev. Cell. 12, 45–55 (2007).

[126] A. Girard, R. Sachidanandam, G. J. Hannon, M. A. Carmell, A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature. 442, 199–202 (2006).

[127] H. Siomi, M. C. Siomi, On the road to reading the RNA-interference code. Nature. 457 (2009), pp. 396–404.

[128] A. a Aravin, R. Sachidanandam, A. Girard, K. Fejes-toth, G. J. Hannon, Developmentally Regulated piRNA Transposon Control. Science (80-.)., 744–747 (2007).

[129] J. Brennecke, A. A. Aravin, A. Stark, M. Dus, M. Kellis, R. Sachidanandam, G. J. Hannon, Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. Cell. 128, 1089–1103 (2007).

[130] S. Kuramochi-Miyagawa, T. Watanabe, K. Gotoh, Y. Totoki, A. Toyoda, M. Ikawa, N. Asada, K. Kojima, Y. Yamaguchi, T. W. Ijiri, K. Hata, E. Li, Y. Matsuda, T. Kimura, M. Okabe, Y. Sakaki, H. Sasaki, T. Nakano, DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev. 22, 908–917 (2008).

[131] C. D. Landry, E. R. Kandel, P. Rajasethupathy, New mechanisms in memory storage: piRNAs and epigenetics. Trends Neurosci. 36, 535–542 (2013).

[132] T. Watanabe, S. I. Tomizawa, K. Mitsuya, Y. Totoki, Y. Yamamoto, S. Kuramochi-Miyagawa, N. Iida, Y. Hoki, P. J. Murphy, A. Toyoda, K. Gotoh, H. Hiura, T. Arima, A. Fujiyama, T. Sado, T. Shibata, T. Nakano, H. Lin, K. Ichiyanagi, P. D. Soloway, H. Sasaki, Role for piRNAs and noncoding RNA in *de novo* DNA methylation of the imprinted mouse Rasgrf1 locus. Science (80-.). 332, 848–852 (2011).

[133] P. Rajasethupathy, I. Antonov, R. Sheridan, S. Frey, C. Sander, T. Tuschl, E. R. Kandel, A Role for Neuronal piRNAs in the Epigenetic Control of Memory-Related Synaptic Plasticity. Cell. 149, 693–707 (2012).

[134] E. J. Lee, S. Banerjee, H. Zhou, A. Jammalamadaka, M. Arcila, B. S. Manjunath, K. S. Kosik, Identification of piRNAs in the central nervous system. Rna. 17, 1090–1099 (2011).

[135] Y. Ghosheh, L. Seridi, T. Ryu, H. Takahashi, V. Orlando, Characterization of piRNAs across postnatal development in mouse brain. Nat. Publ. Gr., 1–7 (2016).

[136] A. Dharap, V. P. Nakka, R. Vemuganti, Altered expression of PIWI RNA in the rat brain after transient focal ischemia. Stroke. 42, 1105–1109 (2011).

[137] P. Zhao, M. Yao, S. Chang, L. Gou, M. Liu, Z. Qiu, Novel function of PIWIL1 in neuronal polarization and migration via regulation of microtubule-associated proteins. Mol. Brain, 1–12 (2015).

[138] J. Roy, B. Mallick, Investigating piwi-interacting RNA regulome in human neuroblastoma. Genes Chromosom. Cancer. 57, 339–349 (2018).

[139] K. T. Wakisaka, The dawn of pirna research in various neuronal disorders. Front. Biosci. 24, 1440–1451 (2019).

[140] J. A. Erwin, M. C. Marchetto, F. H. Gage, Mobile DNA elements in the generation of diversity and complexity in the brain. Nat. Rev. Neurosci. 15 (2014), pp. 497–506.

[141] A. R. Muotri, V. T. Chu, M. C. N. Marchetto, W. Deng, J. V. Moran, F. H. Gage, Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. Nature. 435, 903–910 (2005).

[142] C. A. Thomas, A. C. M. Paquola, A. R. Muotri, LINE-1 Retrotransposition in the Nervous System. Annu. Rev. Cell Dev. Biol. 28, 555–573 (2012).

[143] A. A. Kurnosov, S. V. Ustyugova, V. I. Nazarov, A. A. Minervina, A. Y. Komkov, M. Shugay, M. V. Pogorelyy, K. V. Khodosevich, I. Z. Mamedov, Y. B. Lebedev, The Evidence for Increased L1 Activity in the Site of Human Adult Brain Neurogenesis. PLoS One. 10, e0117854 (2015).

[144] J. K. Baillie, M. W. Barnett, K. R. Upton, D. J. Gerhardt, T. A. Richmond, F. De Sapio, P. M. Brennan, P. Rizzu, S. Smith, M. Fell, R. T. Talbot, S. Gustincich, T. C. Freeman, J. S. Mattick, D. A. Hume, P. Heutink, P. Carninci, J. A. Jeddeloh, G. J. Faulkner, Somatic retrotransposition alters the genetic landscape of the human brain. Nature. 479, 534–537 (2011).

[145] A. Saxena, D. Tang, P. Carninci, PiRNAs warrant investigation in Rett Syndrome: An omics perspective. Dis. Markers. 33, 261–275 (2012).

[146] M. Schulze, A. Sommer, S. Plötz, M. Farrell, B. Winner, J. Grosch, J. Winkler, M. J. Riemenschneider, Sporadic Parkinson's disease derived neuronal cells show disease-specific mRNA and small RNA signatures with abundant deregulation of piRNAs. Acta Neuropathol. Commun. 6, 58 (2018).

[147] W. Qiu, X. Guo, X. Lin, Q. Yang, W. Zhang, Y. Zhang, L. Zuo, Y. Zhu, C. S. R. Li, C. Ma, X. Luo, Transcriptome-wide piRNA profiling in human brains of Alzheimer's disease. Neurobiol. Aging. 57, 170–177 (2017).

[148] J. Roy, A. Sarkar, S. Parida, Z. Ghosh, B. Mallick, Small RNA sequencing revealed dysregulated piRNAs in Alzheimer's disease and their probable role in pathogenesis. Mol. Biosyst. 13, 565–576 (2017).

[149] W. Sun, H. Samimi, M. Gamez, H. Zare, B. Frost, Pathogenic tau-induced piRNA depletion promotes neuronal death through transposable element dysregulation in neurodegenerative tauopathies. Nat. Neurosci. (2018), doi:10.1038/s41593-018-0194-1.

[150] N. G. Coufal, J. L. Garcia-Perez, G. E. Peng, G. W. Yeo, Y. Mu, M. T. Lovci, M. Morell, K. S. O'Shea, J. V. Moran, F. H. Gage, L1 retrotransposition in human neural progenitor cells. Nature. 460, 1127–1131 (2009).

[151] J. Altman, Autoradiographic investigation of cell proliferation in the brains of rats and cats. Anat. Rec. 145, 573–591 (1963).

[152] F. H. Gage, Adult neurogenesis in mammals. Science (80-.). 364 (2019).

[153] S. Farioli-Vecchioli, A. Mattera, L. Micheli, M. Ceccarelli, L. Leonardi, D. Saraulli, M. Costanzi, V. Cestari, J. P. Rouault, F. Tirone, Running rescues defective adult neurogenesis by shortening the length of the cell cycle of neural stem and progenitor cells. Stem Cells. 32, 1968–1982 (2014).

[154] T. J. Fischer, T. L. Walker, R. W. Overall, M. D. Brandt, G. Kempermann, Acute effects of wheel running on adult hippocampal precursor cells in mice are not caused by changes in cell cycle length or S phase length. Front. Neurosci. 8, 314 (2014).

[155] G. Kronenberg, K. Reuter, B. Steiner, M. D. Brandt, S. Jessberger, M. Yamaguchi, G. Kempermann, Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. J. Comp. Neurol. 467, 455–463 (2003).

[156] G. Kronenberg, A. Bick-Sander, E. Bunk, C. Wolf, D. Ehninger, G. Kempermann, Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus. Neurobiol. Aging. 27, 1505–1513 (2006).

[157] S. Lugert, O. Basak, P. Knuckles, U. Haussler, K. Fabel, M. Götz, C. A. Haas, G. Kempermann, V. Taylor, C. Giachino, Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. Cell Stem Cell. 6, 445–456 (2010).

[158] R. W. Overall, T. L. Walker, O. Leiter, S. Lenke, S. Ruhwald, G. Kempermann, Delayed and transient increase of adult hippocampal neurogenesis by physical exercise in DBA/2 mice. PLoS One. 8, e83797 (2013).

[159] H. van Praag, G. Kempermann, F. H. Gage, Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat. Neurosci. 2, 266–270 (1999).

[160] R. W. Overall, T. L. Walker, T. J. Fischer, M. D. Brandt, G. Kempermann, Different mechanisms must be considered to explain the increase in hippocampal neural precursor cell proliferation by physical activity. Front. Neurosci. 10, 362 (2016).

[161] T. Singer, M. J. McConnell, M. C. N. Marchetto, N. G. Coufal, F. H. Gage, LINE-1 retrotransposons: Mediators of somatic variation in neuronal genomes? Trends Neurosci. 33, 345–354 (2010).

[162] T. hao Bao, W. Miao, J. hong Han, M. Yin, Y. Yan, W. wei Wang, Y. hong Zhu, Spontaneous Running Wheel Improves Cognitive Functions of Mouse Associated with miRNA Expressional Alteration in Hippocampus Following Traumatic Brain Injury. J. Mol. Neurosci. 54, 622–629 (2014).

[163] M. Cosín-Tomás, M. J. Alvarez-López, S. Sanchez-Roige, J. F. Lalanza, S. Bayod, C. Sanfeliu, M. Pallàs, R. M. Escorihuela, P. Kaliman, Epigenetic alterations in hippocampus of SAMP8 senescent mice and modulation by voluntary physical exercise. Front. Aging Neurosci. 6, 51 (2014).

[164] T. Hu, F. J. Zhou, Y. F. Chang, Y. S. Li, G. C. Liu, Y. Hong, H. L. Chen, Y. Bin Xiyang, T. H. Bao, miR21 is Associated with the Cognitive Improvement Following Voluntary Running Wheel Exercise in TBI Mice. J. Mol. Neurosci. 57, 114–122 (2015).

[165] A. Pan-Vazquez, N. Rye, M. Ameri, B. McSparron, G. Smallwood, J. Bickerdyke, A. Rathbone, F. Dajas-Bailador, M. Toledo-Rodriguez, Impact of voluntary exercise and housing conditions on hippocampal glucocorticoid receptor, miR-124 and anxiety. Mol. Brain. 8, 40 (2015).

[166] S. R. Richardson, S. Morell, G. J. Faulkner, L1 Retrotransposons and Somatic Mosaicism in the Brain. Annu. Rev. Genet., 1–27 (2014).

[167] B. Czech, M. Munafò, F. Ciabrelli, E. L. Eastwood, M. H. Fabry, E. Kneuss, G. J. Hannon, piRNA-Guided Genome Defense: From Biogenesis to Silencing. Annu. Rev. Genet. 52, 131–157 (2018).

[168] D. M. Ozata, I. Gainetdinov, A. Zoch, D. O'Carroll, P. D. Zamore, PIWI-interacting RNAs: small RNAs with big functions. Nat. Rev. Genet. 20, 89–108 (2019).

[169] R. Beckervordersandforth, A. Deshpande, I. Schäffner, H. B. Huttner, A. Lepier, D. C. Lie, M. Götz, In vivo targeting of adult neural stem cells in the dentate gyrus by a Split-Cre Approach. Stem Cell Reports. 2, 153–162 (2014).

[170] M. Schouten, P. Bielefeld, L. Garcia-Corzo, E. M. J. Passchier, S. Gradari, T. Jungenitz, M. Pons-Espinal, E. Gebara, S. Martín-Suárez, P. J. Lucassen, H. E. De Vries, J. L. Trejo, S. W. Schwarzacher, D. De Pietri Tonelli, N. Toni, H. Mira, J. M. Encinas, C. P. Fitzsimons, Circadian glucocorticoid oscillations preserve a population of adult hippocampal neural stem cells in the aging brain. Mol. Psychiatry (2019), doi:10.1038/s41380-019-0440-2.

[171] S. M. G. Braun, R. A. C. Machado, S. Jessberger, Temporal Control of Retroviral Transgene Expression in Newborn Cells in the Adult Brain. Stem Cell Reports. 1, 114–122 (2013).

[172] P. Zhang, X. Si, G. Skogerbø, J. Wang, D. Cui, Y. Li, X. Sun, L. Liu, B. Sun, R. Chen, S. He, D. W. Huang, PiRBase: A Web resource assisting piRNA functional study. Database. 2014, 1–7 (2014).

[173] A. G. Torres, O. Reina, S.-O. Attolini, L. Ribas De Pouplana, Differential expression of human tRNA genes drives the abundance of tRNA-derived fragments (2019), doi:10.1073/pnas.1821120116.

[174] L. E. Clarke, S. A. Liddelow, C. Chakraborty, A. E. Münch, M. Heiman, B. A. Barres, Normal aging induces A1-like astrocyte reactivity. Proc. Natl. Acad. Sci. 115, E1896–E1905 (2018).

[175] S. A. Liddelow, K. A. Guttenplan, L. E. Clarke, F. C. Bennett, C. J. Bohlen, L. Schirmer, M. L. Bennett, A. E. Münch, W.-S. Chung, T. C. Peterson, D. K. Wilton, A. Frouin, B. A. Napier, N. Panicker, M. Kumar, M. S. Buckwalter, D. H. Rowitch, V. L. Dawson, T. M. Dawson, B. Stevens, B. A. Barres, Neurotoxic reactive astrocytes are induced by activated microglia. Nature. 541, 481–487 (2017).

[176] J. L. Mignone, V. Kukekov, A. S. Chiang, D. Steindler, G. Enikolopov, Neural Stem and Progenitor Cells in Nestin-GFP Transgenic Mice. J. Comp. Neurol. 469, 311–324 (2004).

[177] S. P. Keam, P. E. Young, A. L. McCorkindale, T. H. Y. Dang, J. L. Clancy, D. T. Humphreys, T. Preiss, G. Hutvagner, D. I. K. Martin, J. E. Cropley, C. M. Suter, The human Piwi protein Hiwi2 associates with tRNA-derived piRNAs in somatic cells. Nucleic Acids Res. 42, 8984–8995 (2014).

[178] G. Viero, L. Lunelli, A. Passerini, P. Bianchini, R. J. Gilbert, P. Bernabò, T. Tebaldi, A. Diaspro, C. Pederzolli, A. Quattrone, Three distinct ribosome assemblies modulated by translation are the building blocks of polysomes. J. Cell Biol. 208, 581–596 (2015).

[179] A. Baser, M. Skabkin, A. Martin-Villalba, Neural Stem Cell Activation and the Role of Protein Synthesis. Brain Plast. 3, 27–41 (2017).

[180] S. Tahmasebi, M. Amiri, N. Sonenberg, Translational control in stem cells. Front. Genet. 10 (2019), , doi:10.3389/fgene.2018.00709.

[181] Sampath, P., Pritchard, D.K., Pabon, L., Reinecke, H., Schwartz, S.M., Morris, D.R., and Murry, C.E. (2008). A Hierarchical Network Controls Protein Translation during Murine Embryonic Stem Cell Self-Renewal and Differentiation. Cell Stem Cell 2, 448–460.

[182] Blanco, S., Bandiera, R., Popis, M., Hussain, S., Lombard, P., Aleksic, J., Sajini, A., Tanna, H., Cortés-Garrido, R., Gkatza, N., et al. (2016). Stem cell function and stress response are controlled by protein synthesis. Nature 534, 335–340.

[183] Ingolia, N.T., Lareau, L.F., and Weissman, J.S. (2011). Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147, 789–802.

[184] Liu, Y., Beyer, A., and Aebersold, R. (2016). On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 165, 535–550.

[185] N. Guzzi, M. Cie Sla, P. Cao, T. Ngoc, E. Hellströ M-Lindberg, A. C. Hsieh, C. B. Correspondence, S. Lang, S. Arora, M. Dimitriou, K. Pimková, M. N. E. Sommarin, R. Munita, M. Lubas, Y. Lim, K. Okuyama, S. Soneji, G. Ran Karlsson, J. Hansson, G. Ran Jö, A. H. Lund, M. Sigvardsson, C. Bellodi, Cell, in press, doi:10.1016/j.cell.2018.03.008.

[186] D. D. P. Tonelli, Y. M. Clovis, W. B. Huttner, Mouse Molecular Embryology. 1092, 31–42 (2014).

[187] Klempin, Oppositional effects of serotonin receptors 5-HT1a, 2, and 2c in the regulation of adult hippocampal neurogenesis. Front. Mol. Neurosci. 3 (2010), doi:10.3389/fnmol.2010.00014.

[188] M. Deo, J. Y. Yu, K. H. Chung, M. Tippens, D. L. Turner, Detection of mammalian microRNA expression by in situ hybridization with RNA oligonucleotides. Dev. Dyn. 235, 2538–2548 (2006).

[189] H. Babu, J. H. Claasen, S. Kannan, A. E. Rünker, T. Palmer, G. Kempermann, A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. Front. Neurosci. 5, 89 (2011).

[190] B. Martynoga, J. L. Mateo, B. Zhou, J. Andersen, A. Achimastou, N. Urbán, D. van den Berg, D. Georgopoulou, S. Hadjur, J. Wittbrodt, L. Ettwiller, M. Piper, R. M. Gronostajski, F. Guillemot, Epigenomic enhancer annotation reveals a key role for NFIX in neural stem cell quiescence. Genes Dev. 27, 1769–1786 (2013).

[191] S. Bruno, Z. Darzynkiewicz, Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Prolif. 25, 31–40 (1992).

[192] M. Pons-Espinal, E. de Luca, M. J. Marzi, R. Beckervordersandforth, A. Armirotti, F. Nicassio, K. Fabel, G. Kempermann, D. De Pietri Tonelli, Synergic Functions of miRNAs Determine Neuronal Fate of Adult Neural Stem Cells. Stem Cell Reports. 8, 1046–1061 (2017).

[193] M. Jaskelioff, F. L. Muller, J. H. Paik, E. Thomas, S. Jiang, A. C. Adams, E. Sahin, M. Kost-Alimova, A. Protopopov, J. Cadiñanos, J. W. Horner, E. Maratos-Flier, R. A. Depinho, Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. Nature. 469, 102–107 (2011).

[194] D. R. M. Seib, N. S. Corsini, K. Ellwanger, C. Plaas, A. Mateos, C. Pitzer, C. Niehrs, T. Celikel, A. Martin-Villalba, Loss of dickkopf-1 restores neurogenesis in old age and counteracts cognitive decline. Cell Stem Cell. 12, 204–214 (2013).

[195] D. W. Huang, B. T. Sherman, R. A. Lempicki, Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13 (2009).

[196] D. W. Huang, B. T. Sherman, R. A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57 (2009).

[197] D. Karagkouni, M. D. Paraskevopoulou, S. Chatzopoulos, I. S. Vlachos, S. Tastsoglou, I. Kanellos, D. Papadimitriou, I. Kavakiotis, S. Maniou, G. Skoufos, T. Vergoulis, T. Dalamagas, A. G. Hatzigeorgiou, DIANA-TarBase v8: A decade-long collection of experimentally supported miRNA-gene interactions. Nucleic Acids Res. 46, D239–D245 (2018).

[198] R. L. Boudreau, P. Jiang, B. L. Gilmore, R. M. Spengler, R. Tirabassi, J. A. Nelson, C. A. Ross, Y. Xing, B. L. Davidson, Transcriptome-wide discovery of microRNA binding sites in Human Brain. Neuron. 81, 294–305 (2014).

[199] H. Dweep, N. Gretz, MiRWalk2.0: A comprehensive atlas of microRNA-target interactions. Nat. Methods. 12, 697 (2015).

[200] Z. Hu, D. Yu, Q. H. Gu, Y. Yang, K. Tu, J. Zhu, Z. Li, MiR-191 and miR-135 are required for long-lasting spine remodelling associated with synaptic long-term depression. Nat. Commun. 5, 3263 (2014).

[201] E. Y. van Battum, M. G. Verhagen, V. R. Vangoor, Y. Fujita, A. A. H. A. Derijck, E. O'Duibhir, G. Giuliani, T. de Gunst, Y. Adolfs, D. Lelieveld, D. Egan, R. Q. J. Schaapveld, T. Yamashita, R. J. Pasterkamp, An Image-Based miRNA screen identifies miRNA-135s as regulators of CNS axon growth and regeneration by targeting Krüppel-like factor 4. J. Neurosci. 38, 0662–17 (2017).

[202] C. Mannironi, J. Camon, F. De Vito, A. Biundo, M. E. De Stefano, I. Persiconi, I. Bozzoni, P. Fragapane, A. Mele, C. Presutti, Acute Stress Alters Amygdala microRNA miR-135a and miR-124 Expression: Inferences for Corticosteroid Dependent Stress Response. PLoS One. 8, e73385 (2013).

[203] O. Issler, S. Haramati, E. D. Paul, H. Maeno, I. Navon, R. Zwang, S. Gil, H. S. Mayberg, B. W. Dunlop, A. Menke, R. Awatramani, E. B. Binder, E. S. Deneris, C. A. Lowry, A. Chen, MicroRNA 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity. Neuron. 83, 344–360 (2014).

[204] D. Zurawek, M. Kusmider, A. Faron-Gorecka, P. Gruca, P. Pabian, J. Solich, M. Kolasa, M. Papp, M. Dziedzicka-Wasylewska, Reciprocal MicroRNA Expression in Mesocortical Circuit and Its Interplay with Serotonin Transporter Define Resilient Rats in the Chronic Mild Stress. Mol. Neurobiol. 54, 5741–5751 (2017).

[205] T. L. Walker, R. W. Overall, S. Vogler, A. M. Sykes, S. Ruhwald, D. Lasse, M. Ichwan, K. Fabel, G. Kempermann, Lysophosphatidic Acid Receptor Is a Functional Marker of Adult Hippocampal Precursor Cells. Stem Cell Reports. 6, 552–565 (2016).

[206] C. W. Cotman, N. C. Berchtold, L. A. Christie, Exercise builds brain health: key roles of growth factor cascades and inflammation. Trends Neurosci. 30, 464–472 (2007).

[207] E. Bruel-Jungerman, A. Veyrac, F. Dufour, J. Horwood, S. Laroche, S. Davis, Inhibition of PI3K-Akt signaling blocks exercise-mediated enhancement of adult neurogenesis and synaptic plasticity in the dentate gyrus. PLoS One. 4, e7901 (2009).

[208] M. J. Chen, A. A. Russo-Neustadt, Exercise activates the phosphatidylinositol 3-kinase pathway. Mol. Brain Res. 135, 181–193 (2005).

[209] Z. Cheng, F. Liu, H. Zhang, X. Li, Y. Li, J. Li, F. Liu, Y. Cao, L. Cao, F. Li, miR-135a inhibits tumor metastasis and angiogenesis by targeting FAK pathway. Oncotarget. 8, 31153–31168 (2017).

[210] B. Xu, T. Tao, Y. Wang, F. Fang, Y. Huang, S. Chen, W. Zhu, M. Chen, hsa-miR-135a-1 inhibits prostate cancer cell growth and migration by targeting EGFR. Tumor Biol. 37, 14141–14151 (2016).

[211] L. Guo, G. Ding, W. Xu, H. Ge, Y. Jiang, X. Chen, Y. Lu, MiR-135a-5p represses proliferation of HNSCC by targeting HOXA10. Cancer Biol. Ther., 1–28 (2018).

[212] X. P. Mao, L. S. Zhang, B. Huang, S. Y. Zhou, J. Liao, L. W. Chen, S. P. Qiu, J. X. Chen, Mir-135a enhances cellular proliferation through post-transcriptionally regulating PHLPP2 and FOXO1 in human bladder cancer. J. Transl. Med. 13, 86 (2015).

[213] H. Pei, Z. Jin, S. Chen, X. Sun, J. Yu, W. Guo, MiR-135b promotes proliferation and invasion of osteosarcoma cells via targeting FOXO1. Mol. Cell. Biochem. 400, 245–252 (2015).

[214] H. Wu, M. Huang, P. Cao, T. Wang, Y. Shu, P. Liu, MiR-135a targets JAK2 and inhibits gastric cancer cell proliferation. Cancer Biol. Ther. 13, 281–288 (2012).

[215] D. M. G. Zubieta, M. A. Hamood, R. Beydoun, A. E. Pall, K. C. Kondapalli, MicroRNA-135a regulates NHE9 to inhibit proliferation and migration of glioblastoma cells. Cell Commun. Signal. 15, 1–12 (2017).

[216] H. van Praag, Exercise Enhances Learning and Hippocampal Neurogenesis in Aged Mice. J. Neurosci. 25, 8680–8685 (2005).

[217] E. P. Moreno-Jiménez, M. Flor-García, J. Terreros-Roncal, A. Rábano, F. Cafini, N. Pallas-Bazarra, J. Ávila, M. Llorens-Martín, Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. Nat. Med. 25, 554–560 (2019).

[218] M. K. Tobin, K. Musaraca, A. Disouky, A. Shetti, A. Bheri, W. G. Honer, N. Kim, R. J. Dawe, D. A. Bennett, K. Arfanakis, O. Lazarov, Human Hippocampal Neurogenesis Persists in Aged Adults and Alzheimer's Disease Patients. Cell Stem Cell. 24, 974-982.e3 (2019).

[219] S. Camandola, M. P. Mattson, Brain metabolism in health, aging, and neurodegeneration. EMBO J. 36, 1474–1492 (2017).

[220] R. S. Balaban, S. Nemoto, T. Finkel, Mitochondria, oxidants, and aging. Cell. 120 (2005), pp. 483–495.

[221] I. Liguori, G. Russo, F. Curcio, G. Bulli, L. Aran, D. Della-Morte, G. Gargiulo, G. Testa, F. Cacciatore, D. Bonaduce, P. Abete, Oxidative stress, aging, and diseases. Clin. Interv. Aging. 13 (2018), pp. 757–772.

[222] G. Jain, A. Stuendl, P. Rao, T. Berulava, T. Pena Centeno, L. Kaurani, S. Burkhardt, I. Delalle, J. Kornhuber, M. Hüll, W. Maier, O. Peters, H. Esselmann, C. Schulte, C. Deuschle, M. Synofzik, J. Wiltfang, B. Mollenhauer, W. Maetzler, A. Schneider, A. Fischer, A combined miRNA–piRNA signature to detect Alzheimer's disease. Transl. Psychiatry. 9, 250 (2019).

[223] P. Lenart, J. Novak, J. Bienertova-Vasku, PIWI-piRNA pathway: Setting the pace of aging by reducing DNA damage. Mech. Ageing Dev. 173, 29–38 (2018).

[224] Y. A. Savva, J. E. C. Jepson, Y. J. Chang, R. Whitaker, B. C. Jones, G. St Laurent, M. R. Tackett, P. Kapranov, N. Jiang, G. Du, S. L. Helfand, R. A. Reenan, RNA editing regulates transposon-mediated heterochromatic gene silencing. Nat. Commun. 4 (2013), doi:10.1038/ncomms3745.

[225] J. G. Wood, B. C. Jones, N. Jiang, C. Chang, S. Hosier, P. Wickremesinghe, M. Garcia, D. A. Hartnett, L. Burhenn, N. Neretti, S. L. Helfand, Chromatin-modifying genetic interventions suppress age-associated transposable element activation and extend life span in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 113, 11277–11282 (2016).

[226] H. Bu, S. Wedel, M. Cavinato, P. Jansen-Dürr, MicroRNA Regulation of Oxidative Stress-Induced Cellular Senescence. Oxid. Med. Cell. Longev. 2017 (2017), , doi:10.1155/2017/2398696.

[227] N. Li, D. J. Bates, J. An, D. A. Terry, E. Wang, Up-regulation of key microRNAs, and inverse down-regulation of their predicted oxidative phosphorylation target genes, during aging in mouse brain. Neurobiol. Aging. 32, 944–955 (2011).

[228] G. Caronia-Brown, A. Anderegg, R. Awatramani, Expression and functional analysis of the Wnt/beta-catenin induced mir-135a-2 locus in embryonic forebrain development. Neural Dev. 11, 9 (2016).

[229] A. Anderegg, H. P. Lin, J. A. Chen, G. Caronia-Brown, N. Cherepanova, B. Yun, M. Joksimovic, J. Rock, B. D. Harfe, R. Johnson, R. Awatramani, An Lmx1b-miR135a2 Regulatory Circuit Modulates Wnt1/Wnt Signaling and Determines the Size of the Midbrain Dopaminergic Progenitor Pool. PLoS Genet. 9, e1003973 (2013).

[230] R. De Gregorio, S. Pulcrano, C. De Sanctis, F. Volpicelli, E. Guatteo, L. von Oerthel, E. C. Latagliata, R. Esposito, R. M. Piscitelli, C. Perrone-Capano, V. Costa, D. Greco, S. Puglisi-Allegra, M. P. Smidt, U. di Porzio, M. Caiazzo, N. B. Mercuri, M. Li, G. C. Bellenchi, miR-34b/c Regulates Wnt1 and Enhances Mesencephalic Dopaminergic Neuron Differentiation. Stem Cell Reports. 10, 1237–1250 (2018).

[231] N. C. Inestrosa, E. Arenas, Emerging roles of Wnts in the adult nervous system. Nat. Rev. Neurosci. 11, 77–86 (2010).

[232] N. UrbÃ¡n, F. Guillemot, Neurogenesis in the embryonic and adult brain: same regulators, different roles. Front. Cell. Neurosci. 8, 396 (2014).

[233] C. Cava, I. Manna, A. Gambardella, G. Bertoli, I. Castiglioni, Potential Role of miRNAs as Theranostic Biomarkers of Epilepsy. Mol. Ther. - Nucleic Acids. 13 (2018), pp. 275–290.

[234] V. R. Vangoor, C. R. Reschke, K. Senthilkumar, L. L. Van De Haar, M. De Wit, G. Giuliani, M. H. Broekhoven, G. Morris, T. Engel, G. P. Brennan, R. M. Conroy, P. C. Van Rijen, P. H. Gosselaar, S. Schorge, R. Q. J. Schaapveld, D. C. Henshall, P. N. E. De Graan, R. J. Pasterkamp, Antagonizing increased miR-135a levels at the chronic stage of experimental TLE reduces spontaneous recurrent seizures. J. Neurosci. 39, 5064–5079 (2019).

[235] L. K. Smith, Y. He, J. S. Park, G. Bieri, C. E. Snethlage, K. Lin, G. Gontier, R. Wabl, K. E. Plambeck, J. Udeochu, E. G. Wheatley, J. Bouchard, A. Eggel, R. Narasimha, J. L. Grant, J. Luo, T. Wyss-Coray, S. A. Villeda, β2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. Nat. Med. 21, 932–937 (2015).

[236] S. A. Villeda, K. E. Plambeck, J. Middeldorp, J. M. Castellano, K. I. Mosher, J. Luo, L. K. Smith, G. Bieri, K. Lin, D. Berdnik, R. Wabl, J. Udeochu, E. G. Wheatley, B. Zou, D. A. Simmons, X. S. Xie, F. M.

Longo, T. Wyss-Coray, Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. Nat. Med. 20, 659–663 (2014).

[237] J.-K. Roh, K.-H. Jung, K. Chu, Adult Stem Cell Transplantation in Stroke: Its Limitations and Prospects. Curr. Stem Cell Res. Ther. 3, 185–196 (2008).

[238] L. Madisen, T. A. Zwingman, S. M. Sunkin, S. W. Oh, H. A. Zariwala, H. Gu, L. L. Ng, R. D. Palmiter, M. J. Hawrylycz, A. R. Jones, E. S. Lein, H. Zeng, A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. 13, 133–140 (2010).

[239] T. L. Walker, G. Kempermann, One mouse, two cultures: Isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. J. Vis. Exp. (2014), doi:10.3791/51225.

[240] G. and F. K. B. J. Paxinos, The Mouse Brain in Stereotaxic Coordinates. 2nd Edition (2001).

[241] P. Bielefeld, M. Schouten, G. M. Meijer, M. J. Breuk, K. Geijtenbeek, S. Karayel, A. Tiaglik, A. H. Vuuregge, R. A. L. Willems, D. Witkamp, P. J. Lucassen, J. M. Encinas, C. P. Fitzsimons, Co-administration of anti microRNA-124 and -137 oligonucleotides prevents hippocampal neural stem cell loss upon non-convulsive seizures. Front. Mol. Neurosci. 12 (2019), doi:10.3389/fnmol.2019.00031.

[242] D. Karolchik, The UCSC Table Browser data retrieval tool. Nucleic Acids Res. 32, 493D – 496 (2004).

[243] A. Kozomara, S. Griffiths-Jones, MiRBase: Annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 42 (2014), doi:10.1093/nar/gkt1181.

[244] RNAcentral: an international database of ncRNA sequences. Nucleic Acids Res. 43, D123–D129 (2015).

[245] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, Basic local alignment search tool. J. Mol. Biol. 215, 403–410 (1990).

[246] R. H. Waterston, K. Lindblad-Toh, E. Birney, J. Rogers, J. F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S. E. Antonarakis, J. Attwood, R. Baertsch, J. Bailey, K. Barlow, S. Beck, E. Berry, B. Birren, T. Bloom, P. Bork, M. Botcherby, N. Bray, M. R. Brent, D. G. Brown, S. D. Brown, C. Bult, J. Burton, J. Butler, R. D. Campbell,

P. Carninci, S. Cawley, F. Chiaromonte, A. T. Chinwalla, D. M. Church, M. Clamp, C. Clee, F. S. Collins, L. L. Cook, R. R. Copley, A. Coulson, O. Couronne, J. Cuff, V. Curwen, T. Cutts, M. Daly, R. David, J. Davies, K. D. Delehaunty, J. Deri, E. T. Dermitzakis, C. Dewey, N. J. Dickens, M. Diekhans, S. Dodge, I. Dubchak, D. M. Dunn, S. R. Eddy, L. Elnitski, R. D. Emes, P. Eswara, E. Eyras, A. Felsenfeld, G. A. Fewell, P. Flicek, K. Foley, W. N. Frankel, L. A. Fulton, R. S. Fulton, T. S. Furey, D. Gage, R. A. Gibbs, G. Glusman, S. Gnerre, N. Goldman, L. Goodstadt, D. Grafham, T. A. Graves, E. D. Green, S. Gregory, R. Guigó, M. Guyer, R. C. Hardison, D. Haussler, Y. Hayashizaki, D. W. LaHillier, A. Hinrichs, W. Hlavina, T. Holzer, F. Hsu, A. Hua, T. Hubbard, A. Hunt, I. Jackson, D. B. Jaffe, L. S. Johnson, M. Jones, T. A. Jones, A. Joy, M. Kamal, E. K. Karlsson, D. Karolchik, A. Kasprzyk, J. Kawai, E. Keibler, C. Kells, W. J. Kent, A. Kirby, D. L. Kolbe, I. Korf, R. S. Kucherlapati, E. J. Kulbokas, D. Kulp, T. Landers, J. P. Leger, S. Leonard, I. Letunic, R. Levine, J. Li, M. Li, C. Lloyd, S. Lucas, B. Ma, D. R. Maglott, E. R. Mardis, L. Matthews, E. Mauceli, J. H. Mayer, M. McCarthy, W. R. McCombie, S. McLaren, K. McLay, J. D. McPherson, J. Meldrim, B. Meredith, J. P. Mesirov, W. Miller, T. L. Miner, E. Mongin, K. T. Montgomery, M. Morgan, R. Mott, J. C. Mullikin, D. M. Muzny, W. E. Nash, J. O. Nelson, M. N. Nhan, R. Nicol, Z. Ning, C. Nusbaum, M. J. O'Connor, Y. Okazaki, K. Oliver, E. Overton-Larty, L. Pachter, G. Parra, K. H. Pepin, J. Peterson, P. Pevzner, R. Plumb, C. S. Pohl, A. Poliakov, T. C. Ponce, C. P. Ponting, S. Potter, M. Quail, A. Reymond, B. A. Roe, K. M. Roskin, E. M. Rubin, A. G. Rust, R. Santos, V. Sapojnikov, B. Schultz, J. Schultz, M. S. Schwartz, S. Schwartz, C. Scott, S. Seaman, S. Searle, T. Sharpe, A. Sheridan, R. Shownkeen, S. Sims, J. B. Singer, G. Slater, A. Smit, D. R. Smith, B. Spencer, A. Stabenau, N. Stange-Thomann, C. Sugnet, M. Suyama, G. Tesler, J. Thompson, D. Torrents, E. Trevaskis, J. Tromp, C. Ucla, A. Ureta-Vidal, J. P. Vinson, A. C. von Niederhausern, C. M. Wade, M. Wall, R. J. Weber, R. B. Weiss, M. C. Wendl, A. P. West, K. Wetterstrand, R. Wheeler, S. Whelan, J. Wierzbowski, D. Willey, S. Williams, R. K. Wilson, E. Winter, K. C. Worley, D. Wyman, S. Yang, S. P. Yang, E. M. Zdobnov, M. C. Zody, E. S. Lander, Initial sequencing and comparative analysis of the mouse genome. Nature. 420, 520–562 (2002).

[247] B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. Nat. Methods. 9, 357–359 (2012).

[248] A. R. Quinlan, I. M. Hall, BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics. 26, 841–842 (2010).

[249] M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15 (2014), doi:10.1186/s13059-014-0550-8.

[250] M. D. Robinson, A. Oshlack, A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 11, R25 (2010).

[251] C. Braccia, M. Pons Espinal, M. Pini, D. De, P. Tonelli, A. Armirotti, A new SWATH ion library for mouse adult hippocampal neural stem cells (2018), doi:10.1016/j.dib.2018.02.062.

List of publications and presentations

List of publications

Bernocchi G, Fanizzi FP, De Pascali SA, Piccolini VM, **Gasperini C**, Insolia V, Bottone MG (2015). Neurotoxic Effects of Platinum Compounds: Studies in vivo on Intracellular Calcium Homeostasis in the Immature Central Nervous System. *Toxics* 2015, doi: 10.3390/toxics3020224.

Pons-Espinal M[*], **Gasperini C[*]**, Marzi MJ, Braccia C, Armirotti A, Pötzsch A, Walker TL, Fabel K, Nicassio F, Kempermann G, De Pietri Tonelli D (2019) MiR-135a-5p Is Critical for Exercise-Induced Adult Neurogenesis. *Stem Cell Reports*. 2019, doi: 10.1016/j.stemcr.2019.04.020.

Insolia V[*], Priori EC[*], **Gasperini C**, Coppa F, Cocchia M, Iervasi E, Ferrari B, Besio R, Maruelli S, Bernocchi G, Forlino A, Bottone MG (2019) Prolidase enzyme is required for extracellular matrix integrity and impacts on postnatal cerebellar cortex development. *J Comp Neurol*. 2019, doi: 10.1002/cne.24735.

Oral presentations

Gasperini C *"Identification and functional investigation of PIWI-pathway in mouse adult neurogenesis"* **M. Fagiolini and T. Hensch Lab, Harvard University**. November 20, 2017. Boston, Massachusetts, USA - Invited speaker

Gasperini C, Pons-Espinal M, Cossu RM, Scarpato M, Gustincich S, De Pietri Tonelli D *"Identification and functional investigation of mammalian piRNA-pathway in adult hippocampal neurogenesis"* **Keystone Symposia on Mobile Genetic Elements and Genome Plasticity**. February 11-15, 2018. Santa Fe, New Mexico, USA – Poster and oral presenter

Gasperini C, Pons-Espinal M , Cossu RM, Mangoni D, Scarpato M, Pascarella G, Carninci P, Gustincich S, De Pietri Tonelli D *"Identification of piRNAs and functional investigation of piRNA-pathway in adult mammalian Neural Progenitor Cells"* **Keystone Symposia on Small Regulatory RNAs**. April 14-18, 2019. Daejeon, South Korea – Poster and oral presenter

Gasperini C, Pons-Espinal M , Cossu RM, Mangoni D, Scarpato M, Pascarella G, Carninci P, Gustincich S, De Pietri Tonelli D *"Identification of piRNAs and functional investigation of piRNA-pathway in adult mammalian Neural Progenitor Cells"* **ABCD Congress 2019**. September 19-21, 2019. Bologna, Italy – Oral presenter

Poster presentations

Insolia V, Coppa F, **Gasperini C**, Maruelli S, Besio R, Forlino A, Bernocchi G, Bottone MG *"Phosphorylated Tau in Purkinje neurons. Neuroarchitectural alterations of cerebellar cortex in prolidase deficient mice"* **61st Congress of the Italian Embryological Group and 36th Congress of the Italian Society of Histochemistry**. June 7-10, 2015. Pisa, Italy – Published on European Journal of Histochemistry : EJH, 59(1), 2537.

Insolia V, Coppa F, **Gasperini C**, Maruelli S, Besio R, Forlino A, Bernocchi G, Bottone MG *"Cerebellar cortex neuroarchitecture is altered in prolidase deficient mice"* **PhD national meeting SINS**. February 26, 2015. Naples, Italy.

Gasperini C, Pons-Espinal M, Cossu RM, Scarpato M, Gustincich S, De Pietri Tonelli D *"Identification and functional investigation of mammalian piRNA-pathway in adult hippocampal neurogenesis"* **Abcam Meeting on Adult Neurogenesis**. May 2-4, 2018. Dresden, Germany.

Gasperini C, Pons-Espinal M, Cossu RM, Scarpato M, Gustincich S, De Pietri Tonelli D *"Identification and functional investigation of mammalian piRNA-pathway in adult hippocampal neurogenesis"* **22nd Biennial Meeting of the International Society for Developmental Neuroscience**. May 22-25, 2018. Nara, Japan.

Gasperini C, Pons-Espinal M, Cossu RM, Scarpato M, Gustincich S, De Pietri Tonelli D *"Identification and functional investigation of mammalian piRNA-pathway in adult hippocampal neurogenesis"* **Brayn Conference**. June 29-30, 2018. Genoa, Italy.

Pons-Espinal M*, **Gasperini C***, Marzi MJ, Braccia C, Armirotti A, Pötzsch A, Walker TL, Fabel K, Nicassio F, Kempermann G and De Pietri Tonelli D *"MiR-135a-5p is critical for exercise-induced proliferation of adult neurogenesis"* **ABCD Congress, From stress response to tissue development and regeneration**. September 28-29, 2018. Pavia, Italy.

Gasperini C, Pons-Espinal M, Cossu RM, Mangoni D, Scarpato M, Pascarella G, Carninci P, Gustincich S, De Pietri Tonelli D *"Identification of piRNAs and functional investigation of piRNA-pathway in adult mammalian Neural Progenitor Cells"* **EMBO Symposia, The Complex Life of RNA**. October 3-6, 2018. Heidelberg, Germany.

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Review Process

Reviewer 1 – Prof. Carlos Fitzsimons

Content: *In this thesis, the candidate centrally evaluates the role of small noncoding RNAs in mammalian adult neurogenesis, using mouse models. In this context, the candidate's main findings are two-fold:*

1. *The Piwi Pathway, active in neural stem/precursor cells, prevents the generation of reactive glia cells in the postnatal hippocampus, possible by controlling protein synthesis;*
2. *The microRNA miR-135a is a key regulator of the effect of physical exercise on adult hippocampal neurogenesis, possible acting on specific targets involved in phosphatidylinositol signaling.*

The findings described and discussed in this thesis are of interest and relevance for a vast audience of (neuro)scientists interested in the regulation of adult hippocampal neural stem cells under and pathological conditions and have implications for a deeper understanding of the cellular changes induced by physical exercise in the hippocampus, which may be related to its beneficial cognitive effects.

Structure: *The thesis is composed of a thorough introduction, including clearly formulated hypotheses and objectives; a materials and methods section with a clear description of the procedures used to generate the results described and discussed in two main experimental chapters; and a final section including general conclusions and future perspectives and implications for the field.*

Evaluation: *The thesis is clearly written in the English language and is easy to follow and read. It describes in detail a sequence of experiments that, using state-of-the-art techniques, starts with the validation and extension of concepts previously demonstrated by the De Pietri Tonelli Lab, continues with a thorough study of the Piwi pathway and one specific microRNA singled out from expression profiling in purified adult neural stem cells, and ends with the validation of a key role of small non-coding RNAs in the regulation of postnatal hippocampal neurogenesis.*

The results described in the two experimental chapters are discussed and put into perspective into a well-structured and interesting final discussion section that integrates them into the pre-existing literature. Finally, a number of interesting perspectives for future experiments are given, which will help to advance the

field.

Conclusion: *The thesis describes, elaborates and extends a number of crucial observations that have been published in the high rank scientific journals of the field by the De Pietri Tonelli Lab. Crucially, It includes a detailed and novel description of the hitherto unknown expression and function of the Piwi pathway in somatic neural stem cells. The novel observations in this thesis are of crucial relevance to understand the regulation of neural stem cell proliferation and differentiation in pathophysiological conditions, i.e. after epileptic seizures and the cellular changes induced by physical exercise in the hippocampus. Given the global prevalence of epileptic syndromes, these observations are of relevance and will be most probably published in first rank international peer-reviewed journals.*

Grade suggestion: *Based on the amount of work presented in this thesis, its perceived relevance and taking into account the evaluation standards applied to doctoral theses at the University of Amsterdam, I firmly support the acceptance of the thesis and the evaluation of Caterina Gasperini as doctoral candidate, and suggest to proceed to the thesis defense.*

Author answer I wish to thank very much Dr. Fitzsimons for the time he dedicated to the critical reading of my thesis and his insightful comments. I am very pleased about his positive comments, the support to my study and for acknowledging its novelty and interest for the (neuro)scientific community.

Reviewer comment #1: Minor corrections suggested: The evaluated version of this thesis contains some format issues, such as irregular or absent page numbering, some typos and minor grammatical mistakes.

Author answer to comment 1: I have revised the manuscript according to the minor corrections suggested. In particular, all the formatting issues have been fixed.

Reviewer comment #2: Conceptually, the reasons to select specifically miR-135a for the studies described in the second section of the thesis, entitled miR-135a, should have been more explicitly presented in the section 2, rationale and aim of the thesis. As it is explained in page 36 that this specific microRNA was singled out as result of profiling experiments, it would have been perhaps better for the logical structure of the thesis to name the second section "Exercise-dependent microRNAs" (or similar) instead of "miR-135a" a title that contrasts in its specificity with the more general title of the first section "The Piwi pathway".

Author answer to comment 2: In chapter 2 (rationale and aim of the thesis),

I better explain and justify why we selected specifically miR-135a for this study. The revised text:

"[...] The rationale of this second part of my project is built on evidence that running stimulates hippocampal NPC proliferation and alters miRNA expression in rodents. In particular, we selected miR-135a among a panel of miRNAs downregulated in running mice compared to resting ones. miR-135a was the only dysregulated miRNA which manipulation was able to reduce (overexpression) or increase (silencing) the proliferation of cultured aNPCs. This, we hypothesized investigating miR-135a, which is involved in running induced neurogenesis, would allow the identification of the most prominent pathways that constrain NPC proliferative potential in the adult mouse hippocampus. [...] "

According to reviewer's suggestion, I have changed the title from "miR-135a" to "Exercise-dependent microRNAs". I agree the new title is more consistent with the one of the first section, "The Piwi pathway". Thanks for this suggestion. Indeed, a general title for this section is more appropriate since we performed microRNAs profiling experiments on sorted cells from the hippocampus of resting and running mice, founding 8 miRNAs downregulated in running mice, compared to the control ones. Then we focused our attention on the three most down-regulated ones, miR-135a, miR-190 and miR-203. Finally, as mentioned before, among these miRNAs, only the manipulation of miR-135a was able to reduce (overexpression) or increase (silencing) the proliferation of cultured aNPCs, thus we decided to investigate deeper this specific miRNA.

Reviewer 2 – Prof. Giuseppe Testa

This is an outstanding thesis, parts of which have already been published in highly relevant journals, so both the PhD student and her supervisor should be highly commended for the reach and relevance of this work.

Not being accustomed to the practices of IIT or the University of Genoa in PhD thesis evaluation, I have listed below some remarks that I hope will be useful for finalizing the process.

Author answer: I thank Dr. Testa for reading my thesis and for his precious suggestions. I am really enthusiastic about his appreciation for this work and I agreed with the remarks he pointed out. Indeed, I have improved the revised version of my thesis according to his comments and fixed the minors. Specific point by point reply follows.

Reviewer Comment #1: the findings that Mili and Mili-dependent piRNAs depletion enhances polysome assembly in neural progenitors are of potentially enormous significance. For this reason, a longer, deeper and sharper discussion would be warranted (it is currently limited to less than one page, at p.31) to flesh out the implications of these findings and also to give the reader the needed intellectual context to evaluate its significance (including with reference to translation regulation in the CNS and, vis a vis, in other stem cell compartments). The same applies to the ncRNAs and mRNAs listed in section 3.6 as downstream of Mili, which are listed without a clear albeit cursory recap of their functions. Author Answer to comment 1: I thank the reviewer for this comment, I am aware that the potential role of the Piwi pathway in translation regulation in aNPCs could have exceptional implications and I agree that it deserves a deeper discussion. Thus, I have strengthened this point in the discussion, chapter 3.6.

"[...]. In 2008, Sampath et al. found that global translation was low in undifferentiated embryonic stem cells compared to embryonic body and that differentiation induced an anabolic switch. The increase in translation in differentiated cells coincides with a significant increase in the content of total RNA ($\sim 50\,\%$), ribosomal RNA ($\sim 20\,\%$), and proteins ($\sim 30\,\%$). Remarkably, differentiation increases polysome density compared to undifferentiated embryonic stem cells (Sampath et al., 2008). in 2016, Blanco et al. showed that also skin stem cells have lower protein synthesis than committed cells and that low translation functionally contributes to their maintenance (Blanco et al., 2016). More recently, it has been observed that low protein synthesis rate in stem cells associates with their low cellular metabolism. Activation for proliferation and commitment to differentiate requires a huge remodeling of cellular metabolism leading to sub-

stantial variations in energy production and consumption, which correlates with changes in the protein synthesis rate (Baser et al., 2017).

In fact, during steady state, transcriptional control is the main determinant of the cellular proteome, whereas during early stages of state transition (such as differentiation), translational control becomes the major determinant (Ingolia et al., 2011). Translational control allows cells to promptly respond to internal and external stimuli, even before a new transcription program starts (Liu et al., 2016). In this view, neurogenesis might also be controlled by protein synthesis rates, since it involves transition of NPCs through multiple stages and requires adaptation to the changing microenvironment, including metabolic switch.

At the mechanism level, Ribosomes are the center of the whole protein synthesis machinery and key for fine-tuning the proteome. Under physiological condition, ribosome abundance is not considered a limiting factor for translation initiation in stem cells, however, studies in Drosophila and mammals suggest that differentiation of stem cells relies on increased ribosomal biogenesis. In their study, Ingolia et al. concluded that an increased expression of ribosomal proteins at early stages of differentiation is required to boost the rate of global translation, observed at later stages (Ingolia et al., 2011).

Consistent with these findings, here we showed that aberrant activation of aNPCs and reduced neurogenesis caused by Mili KD, correlates with an increased expression of ribosomal biogenesis protein. In particular, we found the increased expression of ribosomal RNA subunit 5s and mRNA encoding ribosomal proteins L (RPL), Rpl3a, Rpl17 and Rpl26. These data suggest a role of the Piwi-pathway in the regulation of translation machinery and, possibly, protein synthesis. In addition to ribosomes, it is very likely that additional translational factors contribute to translational control in stem cells. The initiation factors eIFs are responsible for translation initiation, however there are only few studies on their role in stem cells. It has been reported that lack of eIFs in mouse is often embryonic or perinatal lethal and has detrimental effects on stem cells and normal development (reviewed in (Tahmasebi et al., 2019)). Interestingly, here we found that eIF4A, which is required for the binding of the 40S ribosomal subunits to the cap-complex of the mRNA, is one of the predicted piRNA targets in differentiating aNPCs. Among the predicted piRNA targets in aNPCs we found also tRNAs and TEs. Interestingly, tRNA fragments control translation in stem cells [172] and TEs (i.e., L1s) regulate differentiation of adult NPCs [132, 141]. Together, these results strongly suggest an involvement of the Piwi pathway in the regulation of translation machinery [. . .] ”

Reviewer Comment #2: p. 25, section 3.4 It would have been preferable to include one additional shRNA construct targeting Mili, in addition to the

scrambled control, so as to confirm the phenotype, so it would be useful to briefly comment on why this experimental design (i.e. only 1 shRNA) was chosen and to comment on the caution needed in interpretation vis a vis the literature on RNAi kd, so that the student shows full awareness of these issues.

Author Answer to comment 2: I thank the reviewer for raising this crucial point, we spent a lot of time discussing the best strategy to perform Mili KD in aNPCs in vitro and in vivo. These cells rapidly divide in proliferating conditions and a synthetic oligonucleotide, like a siRNA, would be diluted in few days. Moreover, aNPCs are not easy to transfect and our purpose was to obtain a permanent rather than transient silencing effect. Thus, the choice of viral short-hairpin RNA (shRNA) construct was dictated by the need of a stable integration of the artificial RNA molecule into the cell genome, to perform long-term KD of Mili RNA (and protein) in cultured aNPCs. In addition, since the shRNA construct encodes also for a GFP reporter, after the transduction we were able to FACS-sort the cells that were transduced with the virus. This allowed us to obtain "a pure" population of aNPCs to screen for the silencing. We choose a commercially available shRNA against MILI sequence and it appeared to work nicely in vitro. After the virus transduction and the FACS-sorting, Mili mRNA and protein levels were significantly reduced in the cells treated with shMILI compared to the control ones. Within this system we then investigated cells proliferation and differentiation.

In parallel, in order to validate the in vitro result, we used another strategy that we previously used for the miR-135a project in vivo: we designed 5 different single-stranded antisense oligonucleotides (LNA GapmeRs) which are highly potent in catalyze RNase H-dependent degradation of complementary RNA targets. The GapmeRs offer high stability and affinity for targets in vitro as well as in vivo: the incorporation of lock nucleic acids (LNA) into oligonucleotides has been shown to improve sensitivity and specificity for many hybridization-based technologies. Indeed, for miRNAs in situ hybridization (see miR-135a project, Fig. 4.1) we chose probes with the same technology and we obtained valuable results even in the detection of low-abundant miRNAs. We validated the silencing efficiency of the 5 GapmeRs in vitro, by measuring the level of Mili mRNA and protein in proliferating aNPCs 48 and 72 hours after transfection (data not shown in this thesis). We chose the 2 GapmeRs that gave the best silencing effect (named GapmeR1 and GapmeR3) and we injected them in different cohorts of mice. All the in vivo data showed in this thesis have been obtained after the injection of GapmeR1 and the negative control. The experiments with GapmeR3 are still on going.

Reviewer Comment #3: the term epigenetic is used through the text without a definition of the specific meaning or set of meanings relevant for this work,

something that is instead quite important given the multiplicity of meanings attached to this term. Author Answer to comment 3: I completely agree with the reviewer and I apologize for this oversight. I have revised the manuscript and I have inserted in the introduction three paragraph (below) about epigenetics (1.1.2 Epigenetic control of adult neurogenesis and noncoding RNAs).

"[...] Regulation of aNSC fate determination is known to be possible at the transcriptional level, but accumulating evidence indicates that additional control layers, such as epigenetics and noncoding RNAs, are involved in this mechanism. Among the most commonly used definitions, epigenetic is the study of changes in gene function that are mitotically and/or meiotically heritable and that do not involve a change in DNA sequence (Wu and Morris, 2001). In this sense, genotypically identical cells can behave phenotypically different thanks to epigenetic alterations in chromatin organization and/or biochemical changes. The dynamic nature of epigenetic mechanisms provides a crucial layer of gene regulation, controlling adult neurogenesis in response to environmental signals. As shown in Figure 1.4, there are four major categories of epigenetic mechanisms, which function as key regulators of gene expression also in adult neurogenesis: chromatin remodeling, histone modification, DNA methylation and noncoding RNAs (ncRNAs) (Figure 1.4).

Even if the main focus of my thesis will be this last category, a brief overview of the other mechanisms is worth, given their relevance in the regulation of neurogenesis. Several studies have shown how chromatin remodeling due to the many types of histone modifications occurring on different histone residues, contributes to the regulation of neuronal differentiation, survival, and maturation. For example, inhibition of histone deacetylase is able to induce neuronal differentiation of adult hippocampal neural progenitors (Hsieh et al., 2004). Despite this study, the function of individual histone deacetylases in adult neurogenesis is largely unresolved and more studies are required. However, it is tempting to speculate that pharmacological inhibition of their activity might become an effective clinical strategy for treating disorders related to adult neurogenesis, such as cognitive decline, and promote neural repair (Hsieh and Zhao, 2016). Another example is the knockdown of lysine-specific demethylase 1 (LSD1) in cultured adult NPCs and in adult mouse DG, which resulted in a dramatically reduction of neural stem cell proliferation (Sun et al., 2010).

Epigenetic modifications implicated in adult neurogenesis comprise also DNA methylation. This modification is a major epigenetic mechanism for the establishment of parental-specific imprints during gametogenesis and gene silencing of the inactivated X chromosome and retro- transposons (Jaenisch and Bird, 2003), but it has been shown to be crucial also during neurogenesis. Indeed, during the neural induction of embryonic stem cells (ESCs) to NPCs, many

pluripotency genes are methylated and silenced (Mohn et al., 2008). Moreover, DNA methyl-transferases (DNMT) 3a and 3b appeared to be essential for specification of neurons and glia, during the early phase of neurogenesis (Feng et al., 2005), and during later stages of neuronal maturation and function (Feng et al., 2010; Levenson et al., 2006). Another DNMT, the 1, is involved in JAK-STAT signaling to control the timing of when precursor cells switch from neurogenesis to gliogenesis during development. Through a chromatin remodeling process, demethylation of genes in the JAK-STAT pathway leads to an enhanced activation of STATs, which in turn triggers astrocyte differentiation (Fan et al., 2005).

Lastly, among the epigenetic mechanisms, ncRNAs play an essential role in adult neurogenesis. [...] “

Reviewer Comment #4: Minor: there are several typos/ English mistakes throughout, so an accurate proofreading is recommended. I list below just a few examples: p. 25: The reference to Figure 3.2 A is erroneous, as the RNAi experiment is shown in Figure 3.3A p. 29: “target” should be replaced by “targets” p. 55: “cause” should be replaced by “causes”

Author Answer to comment 4: I have revised the manuscript according to the minor corrections suggested. In particular, all the typos and mistakes have been fixed.